Approaches to improve treatment and early diagnosis of Buruli ulcer: the role of local and systemic immune responses

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Daniela Schütte

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Prof. Dr. Gerd Pluschke, Prof. Dr. Daniela Finke und Prof. Dr. Peter Itin

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SUMMARY

Buruli ulcer (BU) hits thousands of individuals every year in over 30 countries worldwide, primarily children in remote areas of sub-Saharan Africa. This devastating necrotizing skin infection is caused by *Mycobacterium ulcerans*, a cytotoxic macrolide producing environmental pathogen. The disease distorts and cripples those affected and has great socio-economic impact on people living in endemic regions. Currently recommended treatment options are surgical excision of the lesion, systemic administration of as rifampicin and streptomycin (R-S) or a combination of both. Clinical diagnosis of BU lesions requires the expertise of a skilled physician or health worker, and proper medical care is expensive, time-consuming or not available at all in many BU endemic regions of Africa. Thus, rapid diagnostic tools as well as improved established or new alternative therapies which are safe, inexpensive and easy to handle in a rural setting are urgently needed and the present work focused on these important issues.

Histopathological hallmark of progressing BU disease is a poor inflammatory response despite clusters of extracellular bacilli inside necrotic subcutaneous areas. We conducted detailed histopathological studies on the efficacy of chemotherapy with R-S to restore the local immune responses in early (nodule and plaque) and late (ulcerative) BU lesions, respectively. In early lesions AFB internalized by macrophages and neutrophils were already found after two to four weeks of treatment and started to display irregular ZN staining after eight weeks. Final clearance of the bacterial load depended on the initial size of clusters and the surrounding necrosis. After eight weeks of R-S therapy ulcerative lesions comprised only mycobacterial debris inside focally distributed mononuclear phagocytes. Local cellular immune responses were re-activated very quickly (after two weeks acute infiltration was already prominent) and developed further during the course of antibiotic therapy, resulting in the formation of ectopic tertiary lymphoid tissue. Granulomas and other lymphoid structures developed both in early and late stage lesions in the course of antibiotic treatment, but only nodules and plaques showed abscessus formation, severe haemorrhages and extensive necrosis after completion of eight to twelve weeks chemotherapy. Administration of R-S is efficacious to cure BU, but immunopathological adverse events due to a chronic overreaction of the

immune system may cause healing retardation. Thus, treatment strategies have to be further improved. Our results demonstrate that histopathology can serve as a valuable tool for efficacy evaluation.

M. ulcerans grows best at temperatures around 30°C and not above 37 °C and this property makes the application of heat a treatment option. We employed the phase change material sodiumacetatetrihydrate which is widely used in commercial pocket heat pads as a heat application system for thermotherapy. Laboratory reconfirmed patients with ulcerative BU lesions were included in a proof of principle study and treated for four to six weeks. Patients with large defects had skin grafting after successful heat treatment while smaller ulcers healed completely without further intervention. Punch biopsies were analysed for histopathological changes and local immunological reactions during heat therapy. While massive cellular infiltration was observed during antibiotic therapy, the extent of total leukocyte infiltration in the lesion did not increase during thermotherapy. This may favour a rapid transition from inflammation to healing, as indicated by the clinical response to heat treatment, which was characterized by an extraordinarily rapid epithelization and healing process. All patients remained relapse-free within twelve months of follow-up suggesting thermotherapy a future treatment option for BU.

ZUSAMMENFASSUNG

Jedes Jahr entwickeln Tausende Menschen in über 30 Nationen weltweit einen Buruli Ulkus (BU). Insbesondere Kinder in abgelegenen Regionen West und Zentral Afrikas sind betroffen. Das Umweltbakterium *Mycobacterium ulcerans* produziert ein zytotoxisches Makrolid und verursacht diese zerstörerische, nekrotisierende Hauterkrankung. Die Krankheit hat gravierende sozio-ökonomische Konsequenzen für die endemischen Gebiete, da die Betroffenen nach lang anhaltenden Krankheitsverläufen oft entstellt und verkrüppelt sind. Die Behandlung besteht gegenwärtig aus der chirurgischen Entfernung der Läsion, systemischer Gabe von Rifampicin und Streptomycin (R-S) oder einer Kombination aus diesen beiden Optionen. Nur gut ausgebildete Ärzte oder Pfleger mit Expertise können BU zuverlässig klinisch diagnostizieren, und eine adäquate medizinische Versorgung ist teuer, zeitaufwendig und in vielen BU endemischen Gebieten Afrikas kaum verfügbar. Aus diesem Grund ist die Entwicklung von einfachen diagnostischen Hilfsmitteln sowie eine Verbesserung der Therapiemöglichkeiten dringend erforderlich. Die vorliegende Arbeit konzentriert sich auf diese wichtigen Themen.

Ein fortschreitender BU zeichnet sich durch eine reduzierte Inflammationsreaktion aus, trotz grosser Klumpen extrazellulärer Bakterien im nekrotischen subkutanen Gewebe. Wir haben histopathologische Studien an frühen (Knoten und Plaques) sowie späten (Ulcera) Läsionen durchgeführt, um die Entwicklung lokaler Immunantworten im Verlauf der R-S Chemotherapie zu erfassen. In frühen Läsionen konnten bereits nach zwei- bis vierwöchiger Behandlung Makrophagen und neutrophile Leukozyten mit internalisierten säurefesten Stäbchen beobachtet werden, welche nach acht Wochen begannen Unregelmässigkeiten in der ZN Färbung aufzuweisen. Die endgültige Beseitigung der Bakterien hing von ihrer ursprünglichen Anzahl und dem Ausmass der sie umgebenden Nekrose ab. Nach acht Wochen R-S Therapie enthielten ulzerierende Läsionen nur noch fokale Ansammlungen von mykobakteriellen Trümmern in mononukleären Phagozyten. Die lokale zelluläre Immunantwort wurde sehr schnell reaktiviert (markante akute Infiltration bereits nach zwei Wochen) und über die Dauer der Antibiotikatherapie fortentwickelt. Sowohl bei frühen als auch chronischen Läsionen bildeten sich Granulome und ektopische tertiäre lymphoide Strukturen während der

Antibiotikatherapie aus. Allerdings konnten nur in Knoten und Plaques noch nach Beendigung der acht- bis zwölfwöchigen Chemotherapie Abzessbildung, heftige Einblutungen und ausgedehnte Nekrose beobachtet werden.
Die Gabe von R-S hat sich als effizientes Heilmittel für BU erwiesen, allerdings scheinen die beobachteten immunpathologischen Vorgänge aufgrund einer chronischen Überreaktion des Immunsystems Heilungsverzögerungen hervorzurufen. Behandlungsstrategien bedürfen daher weiterer Verbesserungen. Unsere Resultate demonstrieren, dass die Histopathologie eine nützliche Methode zur Beurteilung ihrer Wirksamkeit darstellt.

Da *M. ulcerans* am besten bei Temperatuen um die 30°C und nicht über 37°C wächst, stellt die lokale Anwendung von Wärme eine mögliche Behandlungsoption dar. Wir haben das Phasenwechselmaterial Sodiumacetat-trihydrat, welches weitverbreit in kommerziell hergestellten Wärmetaschen genutzt wird, als Wärme-Applikationssystem zur Thermotherapie eingesetzt. Durch Laboranalysen bestätigte BU Patienten mit ulzerativen Läsionen wurden in einer Pilot-Studie vier bis sechs Wochen lang behandelt. Patienten mit grösseren Wunden erhielten zusätzlich eine Hauttransplantation nach erfolgreicher Wärmebehandlung, während kleinere Ulcera ohne weitere Interventionen komplett ausheilten. Stanzbiopsien wurden auf histologische Veränderungen und lokale immunologische Reaktionen während der Behandlung hin untersucht. Im Gegensatz zur Antibiotikatherapie, die mit massiven Infiltraten einhergeht, rief die Thermotherapie keinen Anstieg der Gesamtleukozytenzahl innerhalb der Läsion hervor. Dies scheint einen raschen Wechsel von Entzündungsreaktionen zu Heilungsprozessen zu begünstigen. Unterstützt wird diese Hypothese durch die während der Wärmebehandlung zu beobachtende, aussergewöhnlich schnelle Epithelbildung und Wundheilung. Da in einem Nachsorgezeitraum von zwölf Monaten alle Studienpatienten rezidiv-frei blieben, kann die Behandlung von BU mittels Wärme als potentielle Alternativtherapie betrachtet werden.

ABBREVIATIONS

AFB	Acid-Fast Bacilli
ALES	Aide aux Lépreux Emmaüs Suisse
BCG	Bacillus Calmette-Guèrin
BU	Buruli Ulcer
CD20	Cluster of Differentiation 20
DCs	Dendritic cells
ELISA	Enzyme-Linked Immunoabsorbent Assay
HE	Haematoxylin and Eosin staining
IFN	Interferon
IgG	Immunoglobulin G
IHC	Immunohistochemistry
IS	Insertion Sequence
kD	kilo Dalton
mAb(s)	monoclonal Antibody(s)
MIC	Minimum Inhibitory Concentration
NGO	Non Gouvernemental Organisation
PCR	Polymerase Chain Reaction
PFA	Paraformaldehyde
POD	Prevention Of Disability
PPD	Purified Protein Derivative
R-S	Rifampicin and Streptomycin
STI	Swiss Tropical Institute
TB	Tuberculosis
Th	T helper
TLR	Toll-Like Receptor
WHO	World Health Organization
ZN	Ziehl-Neelsen staining

CHAPTER 1

Introduction

1.1 Epidemiology of *M. ulcerans* infection

Buruli ulcer is a necrotizing skin disease due to infection with *Mycobacterium ulcerans*, a slow growing environmental pathogen producing a potent toxin with cytotoxic properties causing devastating ulcerative lesions. The main burden lies on individuals living in rural areas of sub-Saharan Africa, primarily young children under the age of 15.

M. ulcerans has been isolated for the first time in 1948 by McCallum and colleagues from six Australian patients presenting with unusual skin lesions [1]. Retrospectively, large ulcers described by Sir Robert Cook already in 1897 in the population of northeast Congo were almost certainly caused by *M. ulcerans* [2]. Before the 1980s several countries reported focal areas endemic for Buruli ulcer [3]. The Uganda Buruli Group introduced the name “Buruli” ulcer referring to the first reported cases coming from the Buruli region of Uganda near lake Kyoga [4]. Since then, alarming increases in case numbers have been reported and over 30 countries worldwide are affected [5].

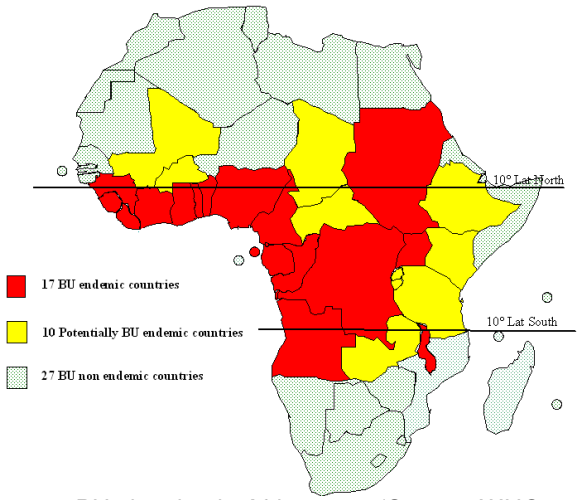

Figure 1. BU situation in Africa 2006 (Source: WHO 2006)

To date, Buruli ulcer is one of the most important human mycobacterioses, outnumbering tuberculosis and leprosy in some countries such as Benin [6]. Efficient surveillance of endemic areas and detection of new cases is difficult in many developing countries and prevalence rates are suspected to be even higher.

1.2 Causative agent

M. ulcerans belongs to a group of mycobacteria called “occasional pathogens” which have the potential to cause disease in humans and animals. Most of those bacteria have an environmental reservoir and infect their hosts only under special circumstances [7]. *M. marinum*, the ancestor of *M. ulcerans*, has a closely related DNA with 99.6% identity. Acquisition of the virulence plasmid and two insertion sequences (IS2404 and IS2606) are the hallmarks of the divergence and species diversification was further driven by acquisition and concomitant loss of DNA [8]. The genome of *M. ulcerans* counts 5.8Mb, carrying over 5000 genes. Two phylogenetic lineages have been identified, the “classical” lineage - including the most pathogenic strains from Africa and Australia - and the “ancestral” lineage – genetically closer to *M. marinum* comprising strains from Asia and South America [9]. African isolates are genetically less heterogeneous than all other known mycobacteria, making epidemiological analyses extremely demanding [10].

M. ulcerans is a slow growing bacillus with generation times between hours and days. Primary cultures are difficult to obtain and may take between several weeks to months to turn positive [11]. The best growing rates are achieved at temperatures around 30°C on Löwenstein-Jensen medium or with the BacTec system [12]. Unlike other members of the *tuberculosis*-complex, *M. ulcerans* does not have a dominating intracellular existence. However, recent studies predominantly in the mouse model, but also in humans showed that bacilli are internalized by phagocytic cells during the initial phase of infection [13,14]. Release of *M. ulcerans* into the tissue occurs due to the action of its exotoxin mycolactone, a polyketide-derived 12-membered ring macrolide [15]. Enzymes required for the synthesis of mycolactone are encoded by a giant 174kb virulence plasmid [16].

Mycolactone has been shown to have both cytotoxic and immunosuppressive activity on leukocytes *in vitro*, leading to cell growth arrest and apoptosis/necrosis [17]. This explains the limited cellular immune responses observed in the core of BU lesions, despite extensive tissue damage and huge clusters of extracellular bacteria. Injection of purified mycolactone into the skin of guinea pigs induces ulceration, whereas mycolactone deficient mutants fail to produce disease in these animals [18].

1.3 Reservoir and transmission

It is assumed that *M. ulcerans* is an environmental organism but the reservoir(s) remain(s) yet unacquainted. BU often strikes individuals living and working in wetlands close to rivers, lakes or other water bodies and increasing incidence rates have been reported in areas where major environmental rearrangements took place [19,20]. Especially in Western Africa the disease is significantly spreading which might be partially due to populations moving into endemic areas as well as increased deforestation, flooding, dam construction or other topographical alterations.

To date the transmission of *M. ulcerans* cannot be fully explained but some sort of skin trauma seems to provide the entry site for infection. Occupational exposure to contaminated soil, for example on plantations or river banks, seems to be associated with development of the disease [21].

1.1.1 **Figure 2.** The Common Brushtail Possum (Trichosurus vulpecula) and its faeces (Source: www.anbg.gov.au)

One hypothesis links the disease to aquatic predatory insects. It has been shown that *M. ulcerans* replicate inside the salivary glands of *Naucoris* spp. and can be transmitted by these biting insects to laboratory mice [22]. Additionally, DNA assigned to *M. ulcerans* has been detected by PCR analysis in other aquatic animals and biofilm formations on aquatic plants [23-25]. In 2008, Portaels et al published the first cultivation of *M. ulcerans* from the environment out of a water strider from Benin [26]. More recently, in an unpublished study in Australia the faeces of possums, small marsupials living in trees (Figure 2), turned out highly positive when tested by real-time PCR. However, no acid-fast bacilli could be detected and culture remained negative.

The discovery of main reservoir(s) and exact mode of transmission of *M. ulcerans* are two important research priorities of the global BU research community.

1.4 The pathology of Buruli ulcer disease

1.4.1 Clinical presentation

The early and less severe forms of BU lesions are mobile subcutaneous nodules (or papules) which are usually painless (Figure 3). Primarily cooler body parts such as the limbs are affected. When left untreated, lesions often become indurated plaques associated with varying degrees of oedema. At this stage bacterial burden is especially high and subcutaneous necrosis spreads relatively fast occasionally involving the underlying fascia and bones [27]. Eventually, plaques turn into an ulcerative lesion, with characteristic cotton wool-like discharge inside the necrotic slough and undermined skin margins where the better part of bacterial burden is located once the central necrosis has been removed [28].

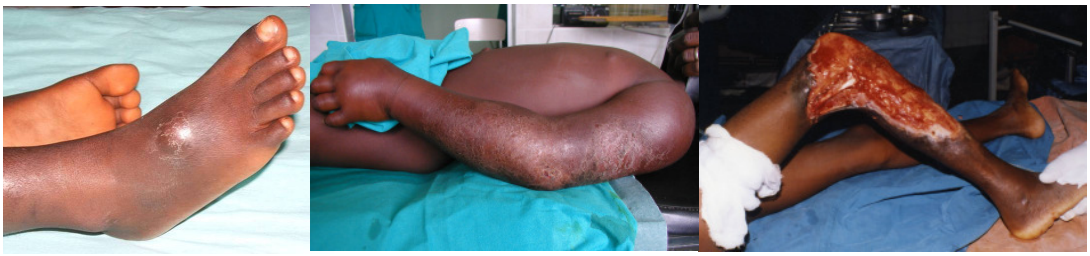

Figure 3. Various stages of *M. ulcerans* infection (Buruli ulcer)

Patients usually present to hospital late, when large plaques or ulcers are established or secondary infections cause additional symptoms such as fever and pain. Therefore, disabling contractures and scarring or even amputation of a limb are common sequelae with great socio-economic consequences for those affected [29].

1.4.2 Histopathological changes

On microscopic level BU presents with a distinct histopathology serving as one of the pillows to confirm clinical diagnosis. All stages have in common a coagulative subcutaneous necrosis, mainly of the adipose and deep dermal connective tissue, which is advancing almost unlimited driving progression of the disease. Huge clusters of extracellular *M. ulcerans* are usually located within the necrotic tissue and

inflammatory responses are mild to absent especially in early lesions [30]. Eventually, all compartments of the affected skin undergo apoptosis/necrosis including vessels, nerves and the epidermal layer, turning structural details into an amorphous eosinophilic coagulum (Figure 4a). As disease progresses into its chronic form, elevated leukocyte infiltration associated with granuloma formation can be observed towards the macroscopically healthy lesion margins [28].

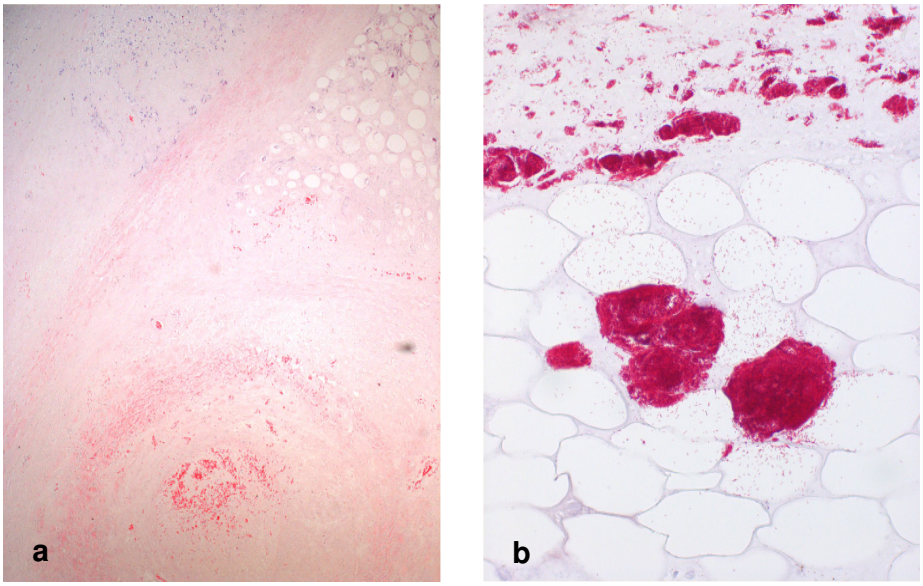

Figure 4. Characteristic BU coagulative necrosis of the subcutaneous tissue (a) with masses of acid-fast bacilli in the adipose layer (b)

Histopathological specimens typically show clumps or microcolonies of acid-fast organisms (Figure 4b), but more recent studies indicate a transient intracellular stage of *M. ulcerans* during the early phase of infection. Phagocytes, occasionally carrying internalized mycobacteria, may be observed at the necrotic rim where some leukocytes managed to infiltrate the tissue despite the immunosuppressive activity of mycolactone [13]. In the mouse foot pad model this phenomenon can be seen more clearly during the very early i.e. pre-nodular stage of the infection. Leukocytes carrying intact bacilli are finally destroyed and release their contents into their environment.

Antibiotic therapy abolishes the characteristic immunosuppression and leads to the development of local strong, organized chronic inflammatory responses and subsequently the destruction of the bacteria (see Chapter 4).

1.4.3 Immune response

BU disease predominantly follows an indolent course, with little inflammatory response and mostly negative *M. ulcerans* or *M. bovis* PPD skin tests [31]. The latter may switch to a positive reaction over time suggesting the development of a systemic response [32,33].

Similar to most mycobacteria, *M. ulcerans* proceeds through an initial intracellular phase, where bacilli are internalized by phagocytes, before transition to the extracellular phase due to cell death caused by mycolactone. This first step may lead to the induction of a Th-1 host response which is ineffective against extracellular pathogens. Suppression of TNF-α in the presence of inflammatory chemokines produced by macrophages may prevent granuloma formation [14]. Several studies on T-helper subset responses have been carried out, because cellular TH-1 responses with high levels of IFN-γ are regarded as crucial for the host defence against mycobacteria [34]. Study protocols implemented were very heterogeneous leading to controversial results. Although one case study reports a shift from Th-1 to Th-2 phenotype during ulceration [35] it is still unclear whether the disease can be associated with a shift in T-helper responses (see Chapter 11).

Mycolactone is not only cytotoxic but at lower concentrations has also immuno-modulatory attributes [36]. Recent evidence suggests that the toxin acts suppressive on antigen presenting leukocytes such as dendritic cells hence disrupting the signal transduction to draining lymph nodes necessary for the activation and homing of lymphocytes [37].

However, people who recovered from BU after surgical excision are not at all protected against recurrences or novel infections. The fact that antibiotic therapy leads to pronounced local leukocyte activation [38] could hint towards a possible “semi-vaccination” based on rifampicin/streptomycin treatment, but long-term follow-ups of patients who recovered solely by administration of antibiotics have not yet been accomplished.

During active disease the majority of *M. ulcerans* are extracellularly located which suggests that antibodies could play a role in immunoprophylaxis and spontaneous healing. Sera from infected individuals have sometimes high antibody titres against

M. ulcerans antigens, not correlated with disease stages [33,39]. Unfortunately, normal BCG vaccination does not provide satisfactory protection [40,41]. Current strategies for better vaccines include repeated or improved BCG vaccination, rational attenuation of a *M. ulcerans* strain and subunit vaccines aimed at immunodominant antigens [42-44]. Vaccination with a compound eliciting the development of neutralizing antibodies against mycolactone is considered an alternative to protein based approaches.

1.5 Diagnostic tools and treatment strategies

1.5.1 Diagnosis

The heterogeneous presentation of BU with its different stages requires an experienced physician for a proper differential diagnosis on clinical grounds.

Developed ulcers are more easily recognized by means of their typical undermined edges and painless nature, although cutaneous leishmaniasis, squamous cell carcinoma or tropical phagedenic ulcer show certain similarities [29]. Examination of swabs or small biopsies taken from the undermined lesion margins is commonly used for confirmation of the clinical diagnosis. Smears can be stained with ZN and assessed for the presence of AFB even in remote areas of resource poor countries [45], but this method has low sensitivity and specificity.

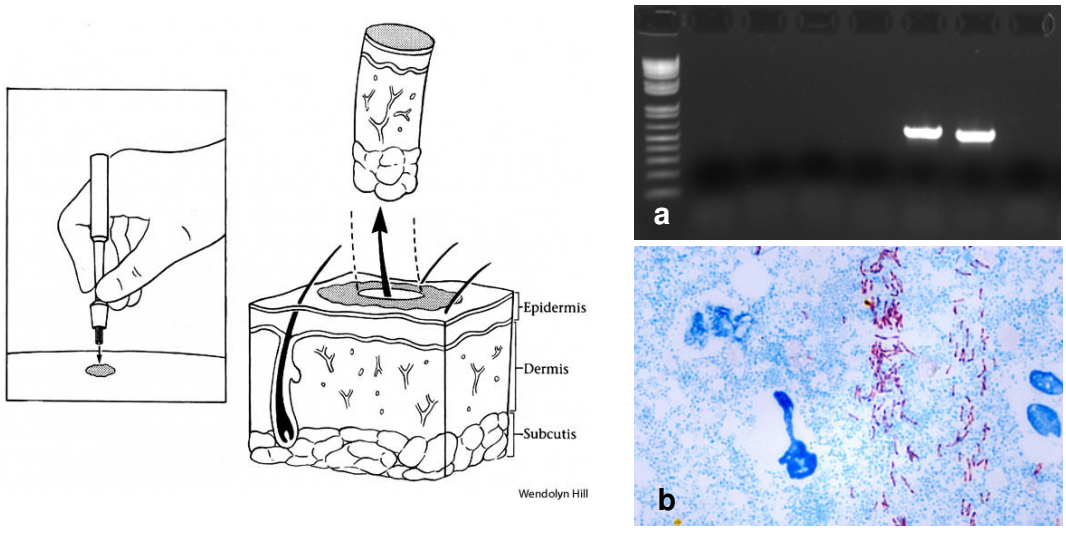

Figure 5. Schematic display of sampling a punch biopsy (source: www.answers.com) and two possible diagnostic applications: PCR of a DNA extract (a) and ZN staining of a smear (b)

PCR based on the amplification of IS2404 and IS2606 is another standard diagnostic tool widely used in better equipped reference laboratories [8]. It is a rapid, sensitive and specific method but requires technical expertise and is not everywhere available.
When it comes to the early nodular stage, clinical diagnosis of BU may even be more often confused with other diseases forming localized swellings such as onchocercoma, lymphadenitis, mycosis or lipoma. The diffuse clinical picture of the oedematous and plaque forms might be mistaken for leprosy, osteomyelitis or psoriasis [29]. In all these cases the skin surface is still intact making invasive sampling for laboratory confirmation of the clinical diagnosis unavoidable. Since recently, punch biopsies are more often used to obtain material which can be used not only for PCR and ZN staining but also for culturing and histopathological assessment (Figure 5). Culture of *M. ulcerans* is a very time-consuming, non-sensitive procedure [11]. Detection of histopathological changes is a reliable diagnostic method but technically demanding and due to the heterogeneous nature of BU lesions often difficult to interpret especially when no AFB can be detected inside the biopsy.
One of the main research priorities of the WHO Global Buruli Ulcer Initiative is the development of a simple and rapid diagnostic test with high sensitivity and specificity that could be used for early identification of cases and would help to improve the implementation of therapeutic interventions and prevention campaigns.

1.5.2 Treatment

During a long period surgical excision of lesions together with subsequent skin grafting was the only available therapeutic intervention. Early non-ulcerative lesions can often be removed without requiring skin grafting preventing the development of disfiguring large ulcers and accompanying deformities. Recurrences after primary surgical excision may occur in up to half the cases due to incomplete removal of the pathogen [46,47]. This invasive therapy and the required long-term care have great economic impact on those affected. Furthermore, in developing countries such as West Africa skilled health care workers and experienced physicians are not commonly in reach and neither are appropriately equipped surgical wards.
Anecdotal reports on antibiotic treatment of BU, especially of complicated cases, have generally been discouraging although *M. ulcerans* is susceptible to rifampicin, some aminoglycosides, macrolides and quinolones *in vitro* [48,49]. In humans, both

clofazamine [50] and cotrimoxacole [51] yielded no effect, and the combination rifampicin/dapsone had limited success on ulcerative lesions [52]. In 2002, Dega et al could show that combined rifampicin and amikacin administration over 12 weeks was sufficient to cure *M. ulcerans* infection in mice [53] and unpublished data from several health centres in Africa reported promising efficacy in patients. Based on these events, WHO launched provisional guidelines on antibiotic therapy of BU patients with rifampicin and streptomycin alone or in combination with surgery in 2004 [54]. One year later Etuaful et al could show that already after four weeks of therapy with this combination culture was rendered negative [55]. A study among patients treated with antibiotics for eight weeks in Benin could categorize 96% of patients as successfully treated. Nevertheless, in about half the cases additional surgery and subsequent skin grafting had to be performed [56]. Recent studies in mice indicate that streptomycin, which has to be injected daily and may cause severe side-effects, could possibly be replaced by the orally administered clarithromycin [57]. Nevertheless, new anti-mycobacterial compounds which are safe, cheap and easy-to-administer are urgently needed as rifampicin is also one out of four frontline drugs in the combat against TB infection.

In the mid-seventies of the last century a small clinical trial was conducted on the local application of heat onto lesions to cure the disease [58]. Although results were quite encouraging, the electricity dependent and complicated device based on water circulation to maintain a constant 40°C made this approach inoperative for rural African regions. Recently, a proof-of-principal trial was accomplished reviving the idea of curing with heat by using bags filled with a cheap, non-hazardous phase-change material (see Chapter 7). First results are very promising as lesions healed rapidly and patients remained free of relapse for over 12 months already, which indicates this strategy to be a possible alternative or supplementary treatment modality in the near future.

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CHAPTER 2

Goal and Objectives

2.1 Goal

To investigate current treatment strategies and future anti-mycobacterial drugs as well as possible alternative diagnostic tools to improve the health care situation of individuals infected with *Mycobacterium ulcerans* in remote African countries.

2.2 Objectives

1. To examine local immunological and histopathological changes during antibiotic therapy of the different stages of Buruli ulcer
2. To compare established treatment modalities and novel therapeutic strategies through local histological processes
3. To assess anti-mycobacterial activity of newly designed bactericidal compounds *in vitro*
4. To investigate the potential of monoclonal antibodies against surface antigens of *M. ulcerans* to develop a new diagnostic tool

CHAPTER 3

Local Activation of the Innate Immune System in Buruli Ulcer Lesions

Elisabetta Peduzzi[1,4], Célia Groeper[2,4], Daniela Schütte[1], Paul Zajac[2], Simona Rondini[1], Ernestina Mensah-Quainoo[3], Giulio Cesare Spagnoli[2], Gerd Pluschke[1] and Claudia Andrea Daubenberger[1]

[1]Swiss Tropical Institute, Department of Medical Parasitology and Molecular Immunology, Basel, Switzerland

[2]Institut für Chirurgische Forschung und Spitalmanagement, Basel University Hospital, Basel, Switzerland

[3]Ghana Health Service, Ministry of Health, Ghana

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Abstract

Buruli ulcer (BU) caused by Mycobacterium ulcerans is a chronic necrotizing disease of the skin and the underlying soft tissue. Fat tissue necrosis accompanied by minimal inflammation is considered the most reliable histopathologic feature of BU. There may be a constant influx of inflammatory cells to the sites of active infection but these are thought to be killed by mycolactone, a polyketide toxin produced by **M. ulcerans**, through apoptosis and necrosis. Here we describe the spatial correlations between mycobacterial load and the expression of dendritic cell (DC) surface markers (cluster of differentiation (CD) 83, CD11c, and CD123), the Toll-like receptor (TLR) 9 and pro- and anti-inflammatory cytokines (IL-8, IL-6, tumor necrosis factor-alpha (TNF-α), IFN-α, IL-12p40, IL-10, and IFN-γ) within BU lesions. Although IL-8, IL-6, and TNF-α messenger RNA (mRNA) was detectable by real-time PCR in all lesions, the expression of the other cytokines was only found as small foci in some lesions. Correlations of the distribution of mRNA encoding the activation marker CD83 and the DC subset markers CD123 and CD11c indicate that both activated plasmacytoid and myeloid dendritic cells were present in the lesions. Results suggest that *M. ulcerans* specific immune responses may develop once therapeutic interventions have limited the production of mycolactone.

Abbreviations

BU, Buruli ulcer; CD, cluster of differentiation; DC, dendritic cell; mRNA, messenger RNA; P-DC, plasmacytoid dendritic cell; TLR, Toll-like receptor; TNF-α, tumor necrosis factor-alpha

Introduction

Buruli ulcer (BU) caused by *Mycobacterium ulcerans* is a chronic necrotizing disease of skin and soft tissue. Generally it manifests initially as firm, non-tender, subcutaneous nodules, probably at the sites of penetrating skin trauma (pre-ulcerative stage). Subsequently, these areas become fluctuant, followed by the formation of an ulceration with undermined edges (ulcerative stage). Ulcers can be extensive, involving more than 10% of the patient's skin surface (Johnson *et al.*, 2005). Subcutaneous fat is particularly affected, but underlying bone may also become involved in advanced cases. In BU lesions clumps of extracellular acid-fast bacilli surrounded by areas of necrosis are found. Fat tissue necrosis accompanied by minimal inflammation is considered the most reliable histopathologic feature of BU (Hayman and McQueen, 1985; Hayman, 1993; Guarner *et al.*, 2003). In late stages of the disease, intralesional influx of leukocytes and granulomatous responses in the dermis and panniculus has been described. If left untreated, spontaneous healing of BU lesions can occur after extended periods of progressive ulceration (Asiedu *et al.*, 2000). Traditionally, BU is treated by wide surgical excision, drug therapy has been considered ineffective, but recent data suggest that combinations of anti-mycobacterial antibiotics can support or replace surgical treatment (Etuaful *et al.*, 2005). Provisional World Health Organization (WHO) guidelines now recommend the use of rifampicin and streptomycin for the treatment of BU (http://www.who.int/buruli/information/antibiotics/en/index1.html).

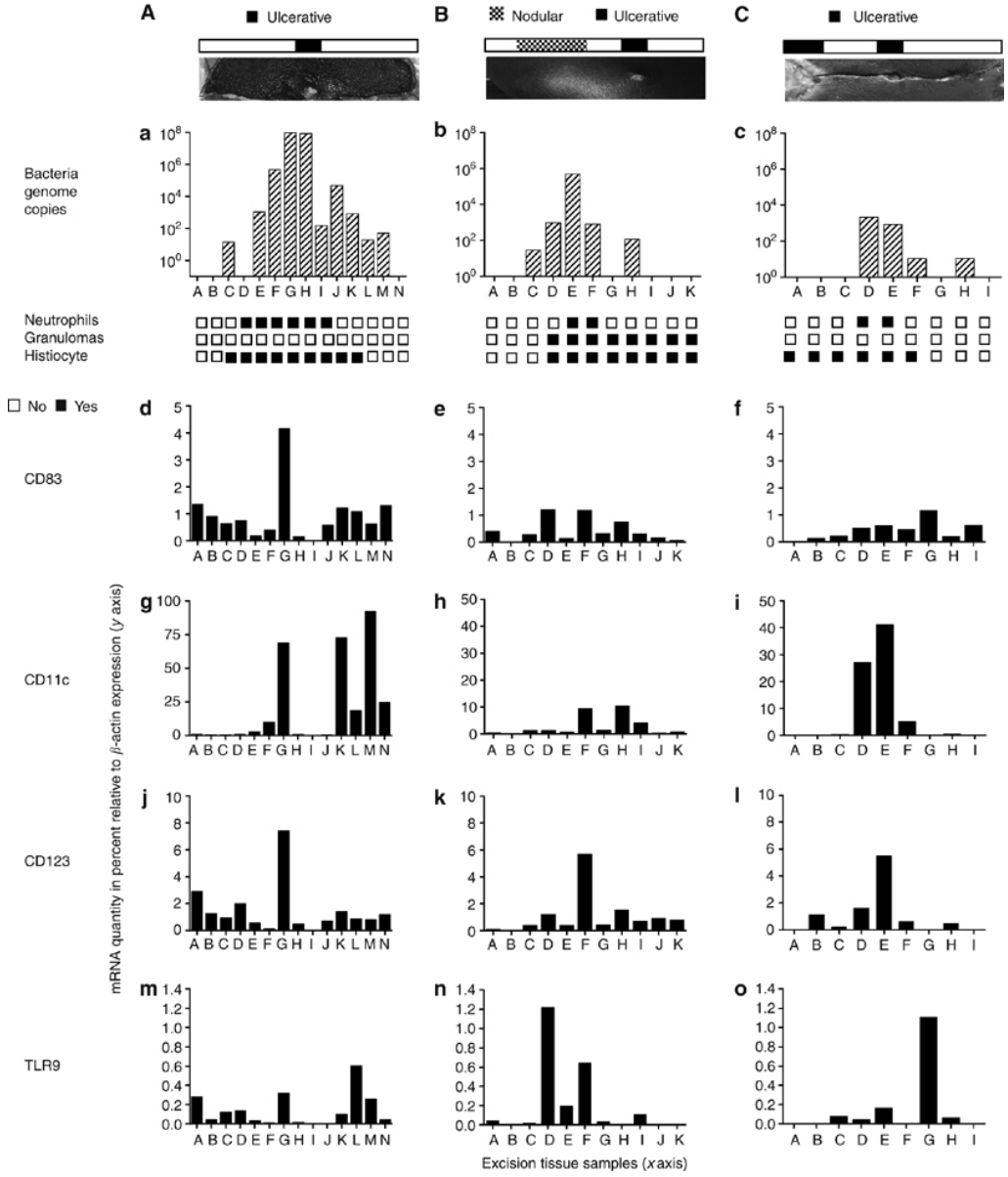
M. ulcerans is unique among mycobacterial pathogens in that it is mainly extracellular and produces a plasmid-encoded toxin with a polyketide-derived macrolide structure, named mycolactone (Stinear *et al.*, 2004). Mycolactone is believed to play a central role in determining the extracellular localization of the bacteria and modulation of immunological responses to *M. ulcerans* (Adusumilli *et al.*, 2005). Observations in rodents experimentally infected with mycolactone producing and mycolactone-negative *M. ulcerans* strains suggested that inflammatory cells are rapidly killed by necrosis when encountering high toxin concentrations. Inflammatory cells more distant from the necrotic center are thought to be killed via apoptosis resulting in extracellular bacteria surrounded by an area of coagulation necrosis. In contrast, granulomatous lesions with strong self-healing tendencies were observed with mycolactone-negative mutants (Oliveira *et al.*, 2005).

Intrigued by the described lack of inflammatory responses in BU lesions, we have analyzed the impact of *M. ulcerans* infection on the activation of the skin innate immune system, including dendritic cells (DC). Here we describe the spatial correlations between bacterial load and the expression of DC-surface markers (cluster of differentiation (CD)83, CD11c, and CD123), the intracellular receptor Toll-like receptor (TLR)9 and pro- and anti-inflammatory cytokines (IL-8, IL-6, tumor necrosis factor-alpha (TNF-α), IFN-α, IL-12p40, IL-10, and IFN-γ) within BU lesions.

Results

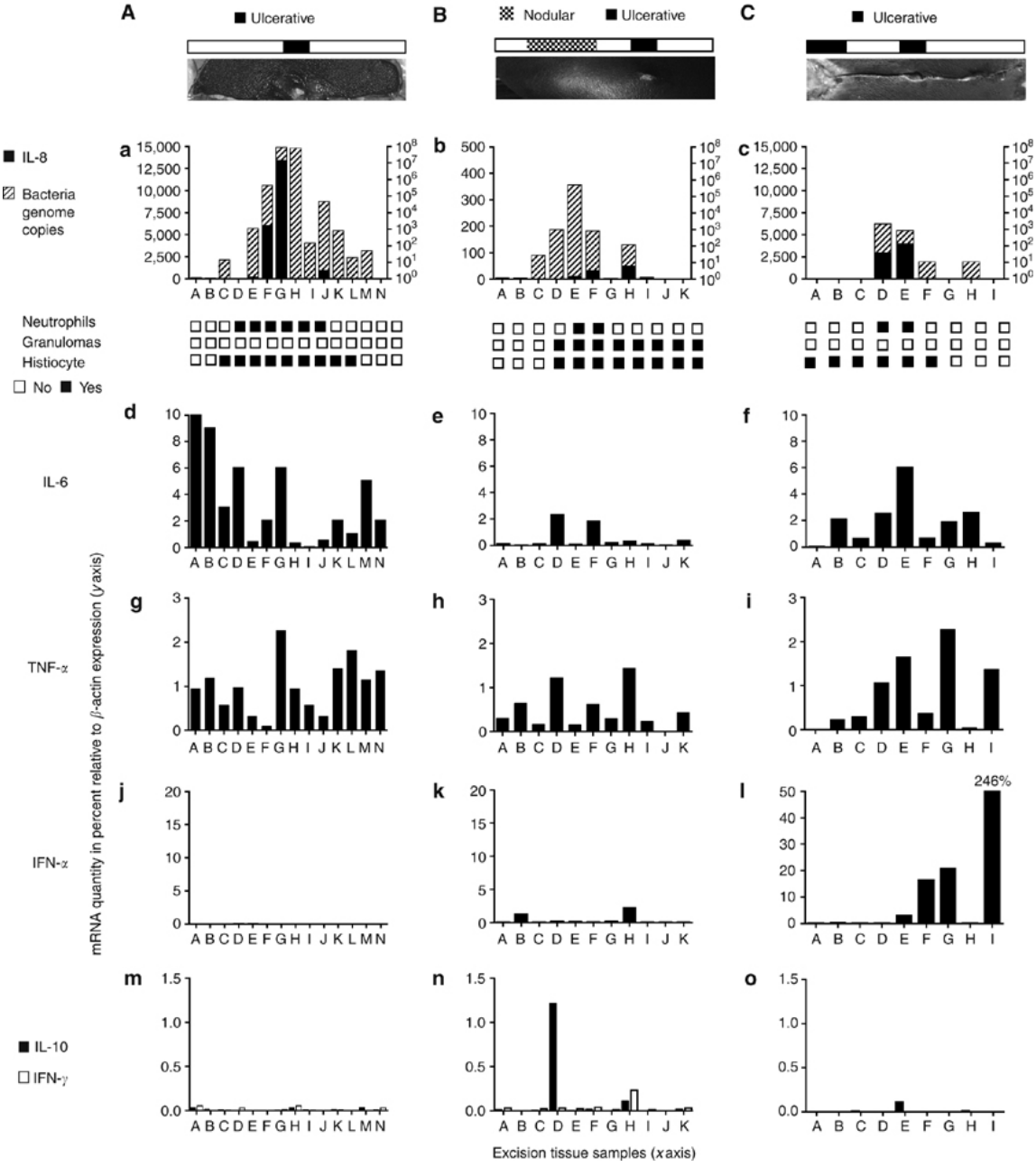
Quantitative real-time PCR was used to determine the spatial distribution of messenger RNA (mRNA) encoding cytokines and cell-surface markers of the innate immune system within surgically excised early ulcerative BU lesions of three selected patients. Histopathological changes and *M. ulcerans* DNA levels in the same tissue samples have been described previously (Rondini *et al.*, 2006). A summary of these data is provided in Figures 1 and 2 for direct comparison with the distribution of cytokine and DC marker mRNA.

Figure 1.



Spatial localization of bacterial load and DC markers in BU lesions. Patients A, B, and C excisions with tissue samples: A–N, A–K, A–I; respectively. Relative quantity of mRNA for the surface markers (d–f) CD83, (g–i) CD11c, (j–l) CD123, and (m–o) intracellular receptor TLR9 expressed in percent relative to β-actin gene expression. (a–c) M. ulcerans DNA load and histopathological changes of the excisions (Rondini et al., 2006).

Figure 2.



Distribution of bacterial load and cytokine mRNA in BU excisions. Patient A, B, and C excisions with tissue samples: A–N, A–K, A–I; respectively. Relative quantity of mRNA for the cytokines (**a–c**) IL-8, (**d–f**) IL-6, (**g–i**) TNF-α, (**j–l**) IFN-α, (**m–o**) IL-10 and IFN-γ expressed in percent relative to β-actin gene expression. (**a–c**) *M. ulcerans* DNA load and histopathological changes of the excisions (Rondini *et al.*, 2006).

Distribution of DC marker mRNA

Figure 1 shows the spatial pattern of mRNA encoding the cell-surface marker CD83, CD11c, CD123, and the intracellular receptor TLR9. Percent values normalized to β-actin mRNA are provided. In all three patients CD83 (Figure 1d–f) and CD123 mRNA (Figure 1j–l) was detectable along the entire lesions. Relative levels ranged from 0 to 4.2% (as compared to 0.2±0.1% in normal skin) and from 0 to 7.4% (0.2±0.2% in normal skin), respectively. CD11c (Figure 1g–i) and TLR9 mRNA (Figure 1m–o) showed a more focal distribution with relative levels ranging from 0 to 92% (0.4±0.2% in normal skin) and 0–1.2% (<0.01% in normal skin), respectively. For all four markers peak values were thus much higher than in normal skin. In many cases peaks were located close to foci of M. ulcerans DNA (sample G in patient A, samples D and F in patient B, and samples D and E in patient C).

Distribution of cytokine mRNA

Expression of cytokines with pro-inflammatory or anti-inflammatory activity was analyzed (Figure 2). IL-8, IL-6, and TNF-α mRNA was detectable in all three BU lesions, albeit in different amounts and in markedly different spatial patterns. Peaks of the relative levels of IL-8 mRNA were associated with the ulcerations and the histological detection of neutrophils (Figure 2a–c). In contrast, location of the relative peaks of IL-6 mRNA with respect to the location of ulcerations and peaks of *M. ulcerans* DNA varied markedly between lesions. In patient A, the relative IL-6 mRNA levels were highest at the less affected borders of the excised tissue, in patient B it was peaking at the nodular pre-ulcerative lesion and in patient C at and around the ulceration (Figure 2d–f). TNF-α mRNA was broadly distributed over the lesion. Relative peak levels of IL-8, IL-6, and TNF-α mRNA (130.395, 10, and 2.3%, respectively) were dramatically higher than the levels found in normal skin (4.7±5.1, 0.1±0.1, and 0.1±0.1%, respectively).

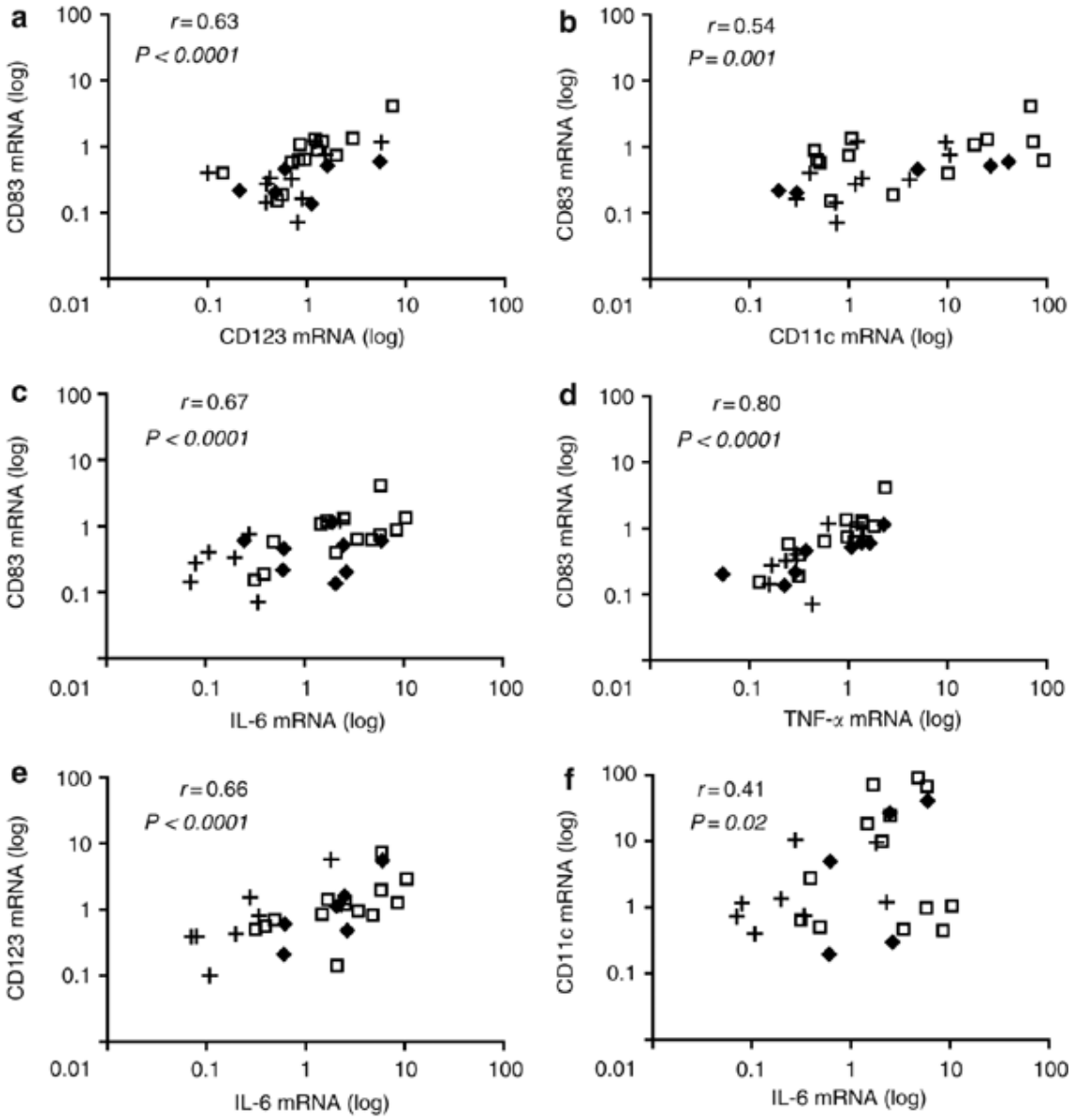
Like in normal skin, IL-12p40 mRNA levels were below the detection limit in all three BU lesions analzsed (<0.03%) (data not shown). In contrast, IFN-α, IFN-γ, and IL-10 mRNA, also undetectable in normal skin, was found at least in one of the three analyzed lesions in spatially highly restricted foci. IL-10 and IFN-γ mRNA was detected only in patient B (Figure 2n). Although a peak of IL-10 mRNA was associated with the secondary non-ulcerated nodule (peak value 1.2%), IFN-γ

mRNA was found in one sample close to the small ulceration and the associated granulomas (peak value 0.2%) (Figure 2n). Significant levels of IFN-α mRNA were primarily found in patient C (Figure 2j–l), peaking towards the right margin of the excised tissue (Figure 2l, peak value in sample I, 246%).

Correlations of the spatial distribution of mRNA species

The spatial mRNA distributions of the two DC subset markers CD11c and CD123 were positively correlated with that of the cellular maturation marker CD83 (CD83 *vs* CD123: r=0.63, P<0.0001; CD83 *vs* CD11c: r=0.54, P=0.001; Figure 3a and b). Also strong positive correlations of the distribution of CD83 with IL-6 and TNF-α were observed (r=0.67, P<0.0001, and r=0.80, P<0.0001, respectively; Figure 3c and d). Correlation of IL-6 expression with CD123 was tighter (r=0.66, P<0.001; Figure 3e) compared with CD11c (r=0.41, P=0.02; Figure 3f). The correlation of both DC subset markers with TNF-α expression was moderate (CD11c *vs* TNF-α: r=0.50, P=0.002; CD123 *vs* TNF-α: r=0.48, P=0.004; data not shown). There was no indication of a correlation between IFN-α and CD123, CD83, or CD11c (data not shown).

Figure 3.

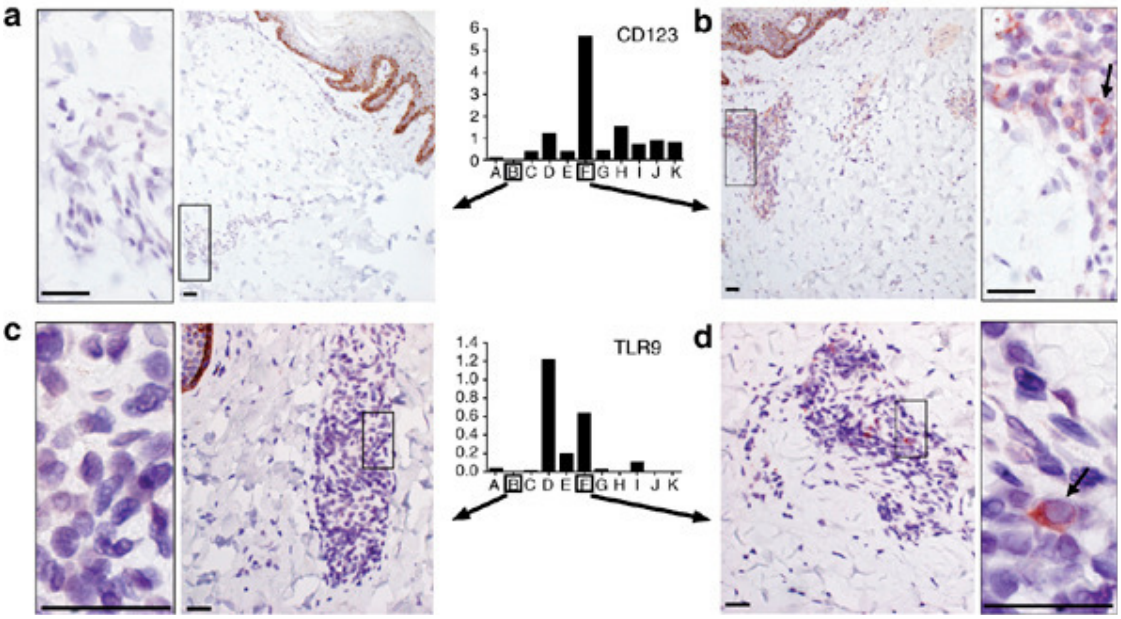


Correlations of DC markers and cytokine expression. Patient A (●), B (◆), and C (+). Spearman's correlations of the mRNA spatial distribution in the excisions are given. (**a**) CD123 versus CD83; (**b**) CD11c versus CD83; (**c**) IL-6 versus CD83; (**d**) TNF-α versus CD83; (**e**) IL-6 versus CD123; (**f**) IL-6 versus CD11c. Spearman's correlations with an *r* ranging from 0.4 to 0.6 and *P*-value of <0.05 are moderate positive; with *r*>0.6 and *P*<0.05 are positive to strong positive. Each point represents the values of one tissue sample.

Immunohistochemical detection of CD123 and TLR9-positive cells

Results of quantitative real-time PCR and immunohistochemistry were highly associated, that is the relative numbers of CD123 and TLR9-positive cells were consistent with the mRNA levels detected by real-time PCR (Figure 4). In the thin sections of lesions positive for CD123 and TLR9 mRNA, CD123, and TLR9 antibodies stained cells with plasmacytoid features (inset Figure 4b and d).

Figure 4.



Immunohistochemical analysis of P-DC recruitment to BU excisions. Immunohistochemical stainings in thin sections of tissues samples B and F of patient B. (**a**, **b**) CD123 staining (original magnification ×100, inset original magnification × 400) and (**c**, **d**) TLR9 staining (original magnification ×200, inset original magnification ×1,000), bars=40 μm.

Discussion

Under homeostatic conditions, cutaneous DCs include epidermal Langerhans cells and interstitial/dermal DCs that are of myeloid origin (Kupper and Fuhlbrigge, 2004). Our real-time PCR and immunostaining data indicate that in addition to the CD11c-positive CD123-negative myeloid DCs (Colonna *et al.*, 2004), CD123-positive plasmacytoid DCs (P-DC), are present in early ulcerative lesions. P-DC are of lymphoid origin (Colonna *et al.*, 2004), CD11c-negative and known to be recruited to diseased skin in conditions such as systemic lupus erythematosus, atopic dermatitis, psoriasis vulgaris, and contact dermatitis (Wollenberg *et al.*, 2002; Bangert *et al.*, 2003; Nestle *et al.*, 2005).

One of the surface molecules upregulated upon DC activation and maturation is CD83 (Lechmann *et al.*, 2002). Although CD83 is also expressed on activated human B and T cells and a subpopulation of activated monocytes (Lechmann *et al.*, 2002), the observed correlations of mRNA expression between CD83 and CD123, or CD11c indicated, that both P-DC and myeloid DC were activated in the BU lesions. The distribution of mRNA encoding the highly expressed pro-inflammatory cytokines IL-6 and TNF-α was also strongly correlated with the activation marker CD83. Expression of IL-6 was additionally strongly correlated with that of CD123, indicating that activated P-DC may represent the major source of IL-6 expression in the BU lesions. In contrast to myeloid DC, P-DC express TLR7 and TLR9 but lack TLR2, TLR3, TLR4, and TLR5. In the majority of patient samples analyzed, expression of TLR9 and CD123 mRNA was consistent, supporting the presence of P-DC in BU lesions (Figure 1). Signalling through TLR7 and TLR9 results in P-DC activation to secrete large amounts of type I IFN and moderate amounts of TNF-α and IL-6 (Colonna *et al.*, 2004). In contrast to IL-6, no correlation between CD123 and IFN-α mRNA was observed. IFN-α expression by P-DC seems to be variable; whereas P-DC activation by TLR9 in response to viruses results in secretion of large amounts of IFN-α (Colonna *et al.*, 2004), during the development of psoriatic phenotype IFN-α expression by P-DC seems to be only an early and transient event (Nestle *et al.*, 2005). Consistent with published data (Prevot *et al.*, 2004; Kiszewski *et al.*, 2006; Phillips *et al.*, 2006), TNF-α and IL-8 mRNA levels were, like those of IL-6 and IFN-α mRNA, much higher in the BU lesions than in normal skin. As moderate correlation between DC markers and TNF-α mRNA was observed, TNF-α mRNA expression may

be in part associated with other cell types, like monocytes, activated T cells, or natural killer cells.

The mechanism of immune protection in *M. ulcerans* remains unclear. Evidence from genetic defects in the IFN-γ signalling pathway supports the role of IFN-γ in protection against a range of non-tuberculous mycobacterial disease, including *M. ulcerans* (Ottenhoff *et al.*, 2005). Peripheral blood mononuclear cells from BU patients with active disease showed significantly reduced lympho-proliferation and IFN-γ production in response to stimulation with live or dead *M. bovis Bacillus* Calmette-Guérin, *M. ulcerans*, purified protein derivative of *M. tuberculosis*, isopentenyl pyrophosphate, and non-mycobacterial antigens like reconstituted influenza virosomes (Gooding *et al.*, 2001, 2002, 2003; Prevot *et al.*, 2004; Yeboah-Manu *et al.*, 2006). Prevot *et al.* (2004) showed with semiquantitative PCR analyses that the systemic Th1 downmodulation was mirrored by local, intralesional cytokine profiles. High IFN-γ with low IL-10 mRNA levels were present in early, nodular lesions, and low IFN-γ mRNA levels were detected in late ulcerative lesions (Prevot *et al.*, 2004). Hence, in active *M. ulcerans* disease, the Th1 response seemed to be downregulated both locally and systemically. The presence of IL-6 during T-cell priming may promote Th2 differentiation and simultaneously inhibit Th1 polarization (Diehl and Rincon, 2002). Therefore the close association of CD123 (P-DC) with IL-6 in conjunction with the lack of IFN-α production may favor Th2 development and result in the observed Th1 downmodulation in BU. IL-6 is a pleiotropic cytokine involved in the growth and differentiation of numerous cell types, including those of dermal and epidermal origin (Paquet and Pierard, 1996). In the skin, it is induced in a broad range of dermatotoxic reactions and may be involved in wound healing (Hernandez-Quintero *et al.*, 2006). The presence of high numbers of P-DC in the lesions in the absence of IFN-α gene expression raises also the issue of a tolerogenic role of these cells, as suggested in primary cutaneous melanomas (Vermi *et al.*, 2003).

IL-10 and IFN-γ mRNA was detected in one of the three analyzed BU lesions, where it was present only in highly focal areas. Phillips *et al.* (2006) showed wide variations in IL-10 and IFN-γ mRNA expression among individual skin punch biopsies. Generally, our results demonstrate that expression of cytokines and cell-surface markers can vary considerably within a BU lesion. Therefore, results obtained with

biopsies of BU lesions do not necessarily reflect the overall profile of a lesion. Our comparison of the spatial relationship between bacterial load, DC marker, and pro-inflammatory cytokine mRNA suggests that the presence of clusters of *M. ulcerans* does not exclude innate immune system recruitment to the site of infection. This conclusion is consistent with the hypothesis of Oliveira *et al.* (2005) suggesting a constant influx of neutrophils, monocytes/macrophages, and lymphocytes to active *M. ulcerans* lesions. Potentially, *M. ulcerans* specific immune responses may therefore develop, once a therapeutic intervention, such as a successful antibiotic treatment, is limiting the production of mycolactone.

Materials and Methods

Clinical specimens

Three BU patients with ulcerative lesions, who received standard treatment at the Amasaman Health Centre in the Ga district in Ghana, were enrolled in this study. The standard treatment comprised wide surgical excision including margins of macroscopically healthy tissue followed by skin grafting. BU clinical diagnosis was reconfirmed by IS2404 PCR, microscopic detection of acid-fast bacilli, and observation of characteristic histopathological changes. The distribution of *M. ulcerans* DNA and histopathological examination within the excised tissue samples analyzed here, have been described elsewhere (Rondini *et al.*, 2006). Patient A presented with an ulcerated plaque (ulcer size 4 × 5 cm) on the dorsal aspect of the left upper arm. The central necrotic slough was associated with typical inflammatory cells whereas no granulomas were seen in any zone of the excision. The highest mycobacterial DNA burden was present at the base of the ulcer and decreased towards the margins of the excision (Figure 1a). Patient B presented with a small-ulcerated lesion (∅ 1 cm) and a larger non-ulcerated nodule located about 3 cm apart on the dorsal aspect of the right elbow. The *M. ulcerans* DNA was present with high load within the non-ulcerated nodule and, with lower load, in the ulcerated region. Between these two lesions no bacterial DNA was detected. Granulomas were present across the tissue from nodule to ulcer till the right margin of the excision (Figure 1b). Patient C presented with a small ulcer 5 cm away from a larger ulcer, which was surrounded by scar tissue and showed evidence of previous treatment. *M.*

ulcerans DNA was only present at the base of the small ulcer and no granulomas were detected in the whole excision (Figure 1c). Ethical approval for analyzing patient specimens was obtained from the local ethical review board of the Noguchi Memorial Institute for Medical Research and participants gave their written informed consent. The study was conducted according to the Declaration of Helsinki Principles.

RNA extraction, removal of genomic DNA, and reverse transcription

RNA was extracted from several samples of equal size, each comprising skin and fat tissue, which were obtained from BU patients' excised lesions: patient A, 14 samples (A–N); patient B, 11 samples (A–K); patient C, nine samples (A–I). Samples were disrupted by sonication for 2 minutes (Sonifer® Branson 250, Branson Ultrasonics Corporation, Danbury, CT) and centrifuged for 3 minutes at 10,000 ×*g*. RNA was extracted from the tissue lysate using the RNeasy Mini Kit (Qiagen AG, Basel, CH) and treated with DNase I (Invitrogen, Paisley, UK) to remove genomic DNA.

To synthesize complementary DNA, total RNA was incubated with oligo d(T) for 10 minutes at 65°C, and put on ice. A reaction mixture containing dNTP mix (125 nM), dithiothreitol (10 mM) and reverse transcriptase Moloney murine leukemia virus (200 U) with corresponding first strand buffer was added (Invitrogen, Paisley, UK). The reaction mix was incubated for 60 minutes at 37°C before enzyme inactivation for 5 minutes at 94°C.

Quantitative real-time polymerase chain reaction

Gene transcription was evaluated using the ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA). Primers and probes for six human cytokines (IL-6, IL-8, IL-10, IL-12p40, TNF-α, IFN-α, and IFN-γ), cell-surface proteins (CD11c, CD83, CD123, and TLR9) were used to amplify specific complementary DNA in duplicate, according to the manufacturers instructions (Applied Biosystems, Foster City, CA). The β-actin gene was used as an internal house keeping gene reference. Primers and probes for β-actin, IL-8, IL-12p40, IFN-α, CD11c, CD83, CD123, and TLR9 were obtained from Applied Biosystems. Primer and probes for IL-6 (Hartwig *et al.*, 2002), IL-10 (Giulietti *et al.*, 2001), TNF-α (Razeghi *et al.*, 2001), and IFN-γ (Kammula *et al.*, 1999) were synthesized by Mycrosynth (Balgach, CH).

Having verified that the amplification dynamic remains proportional at all tested dilutions, RNA expression of each surface marker and cytokine was presented as percentage relative to β-actin gene expression. The assays were run in duplicates and the results with a standard deviation >2% were excluded. Correlation analyses were performed in Prism using the Spearman rank correlation coefficient. Mean values of surface markers and regulatory cytokines in six samples of healthy skin tissue are as follow: CD11c (0.4±0.2%), CD123 (0.2±0.2%), CD83 (0.2±0.1%), TLR9 (<0.01%), TNF-α (0.1±0.1%), IL-6 (0.1±0.1%), and IL-8 (4.7±5.1%). IFN-α, IFN-γ, IL-10, and IL-12p40 mRNA were undetectable.

Immunohistochemistry

Tissue samples were fixed overnight in neutral buffered 4% paraformaldehyde, embedded in paraffin according to standard protocol and cut into 5 μm sections using a microtome. After de-paraffinization, sections were re-hydrated through graded alcohols and washed in distilled water. Antigen retrieval was performed by microwave unmasking technique in 10 mM EDTA pH 8.0. Subsequently, endogenous peroxidase was blocked with 0.3% H_2O_2 for 30 minutes and unspecific binding prevented by incubating with blocking serum for 20 minutes at room temperature. CD123 (clone 6H6) and TLR9 (clone eB72-1665) antibodies (eBiosciences, San Diego, CA) were diluted 1:100 and 1:1,000, respectively, in phosphate-buffered saline plus 0.1% Tween-20 and slides incubated in a humid chamber for 1 hour at room temperature. Sections were incubated for 30 minutes at room temperature with the secondary antibody biotin labelled (Vector Laboratories; 1:200 in phosphate-buffered saline). Slides were then labelled with streptavidin horseradish peroxidase conjugate (Vector Laboratories, Vectastain Elite ABC kit) for 30 minutes at room temperature and staining was performed by using Vector NovaRed and hematoxylin (counter stain).

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CHAPTER 4

Development of Highly Organized Lymphoid Structures in Buruli Ulcer Lesions after Treatment with Rifampicin and Streptomycin

Daniela Schütte [1], Alphonse Um-Boock [2], Ernestina Mensah-Quainoo [3], Peter Itin [4], Peter Schmid [5], Gerd Pluschke [1]

[1] Swiss Tropical Institute, Molecular Immunology, Basel, Switzerland

[2] Leprosy Relief Emmaus-Switzerland (ALES) Cameroon, Yaounde, Cameroon

[3] Ghana Health Service, Amasaman, Ghana

[4] University Hospital Basel, Department of Dermatology, Basel, Switzerland

[5] Novartis Institutes of BioMedical Research, Basel, Switzerland

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Abstract

Background: Buruli ulcer caused by *Mycobacterium ulcerans* is an infection of the subcutaneous tissue leading to chronic necrotising skin ulcers. The pathogenesis is associated with the cytocidal and immunosuppressive activities of a macrolide toxin. Histopathological hallmark of progressing disease is a poor inflammatory response despite of clusters of extracellular bacilli. While traditionally wide excision of the infected tissue was the standard treatment, provisional WHO guidelines now recommend an eight week pre-treatment with streptomycin and rifampicin.

Methodology/ Principal Findings: We conducted a detailed immunohistochemical analysis of tissue samples from Buruli patients who received antibiotic treatment. Cellular immune response along with bacterial load and distribution were monitored. We demonstrate that this treatment leads to the development of highly organized cellular infiltration surrounding areas of coagulative necrosis. Diffuse infiltrates, granulomas and dense lymphocyte aggregation close to vessels were observed. Mycobacterial material was primarily located inside mononuclear phagocytes and microcolonies consisting of extracellular rod-shaped mycobacteria were no longer found. In observational studies some patients showed no clinical response to antibiotic treatment. Corresponding to that, one of five lesions analysed presented with huge clusters of rod-shaped bacilli but no signs of infiltration.

Conclusions/ Significance: Results signify that eight weeks of antibiotic treatment reverses local immunosuppression and leads to an active inflammatory process in different compartments of the skin. Structured leukocyte infiltrates with unique signatures indicative for healing processes developed at the margins of the lesions. It remains to be analysed whether antibiotic resistance of certain strains of *M. ulcerans,* lacking patient compliance or poor drug quality are responsible for the absent clinical responses in some patients. In future, analysis of local immune responses could serve as a suitable surrogate marker for the efficacy of alternative treatment strategies.

Synopsis

Buruli ulcer (BU) is a debilitating disease of the skin presenting with extensive tissue destruction and suppression of local host defence mechanisms. Surgical removal of the affected area has been the standard therapy until in 2004 WHO recommended eight weeks treatment with the anti-mycobacterial drugs rifampicin and streptomycin. We performed a detailed histological analysis of the local immune response in biopsies from five children medicated according to WHO provisional guidelines. One patient still revealed all histopathological signatures of an active BU lesion with huge bacterial clusters in areas of fatty tissue necrosis. Different factors can contribute to treatment failure like poor patient compliance and resistant bacterial strains. In four patients, different compartments of the skin presented active immune processes with only limited residues of bacterial material persisting. We demonstrated that antibiotic treatment not only directly controls the infectious agent but is also associated with fulminant host immune responses. Characterization of the healing process in BU due to therapy is highly relevant to increase our knowledge on the impact of treatment strategies to fight the disease.

Introduction

Buruli ulcer (BU) caused by *Mycobacterium ulcerans* is a chronic necrotizing skin disease mainly affecting subcutaneous and adipose tissue [1,2]. The unique pathology of BU is primarily attributed to a plasmid-encoded macrolide toxin, mycolactone [3,4]. Mycolactone has cytopathic and apoptotic activity and is thought to be responsible for local immunosuppression by destroying infiltrating cells [3,5,6]. In animal models, injection of purified mycolactone causes lesions similar to those produced by wild type *M. ulcerans* bacteria [3,7].

BU is considered to be the third most common mycobacterial infection after tuberculosis and leprosy. Clinical lesions usually start as painless subcutaneous nodules that may develop into plaques or oedema. If left untreated, extensive ulcerations with typical undermined edges of the dermis develop. Spontaneous healing can occur, often leaving the patient behind with extensive scarring, retractions and deformities [8-10]. BU has been reported in more than 30 countries worldwide, but rural communities in Western and Central Africa are the worst affected [2]. Areas endemic for BU are associated with stagnant or slow-flowing water bodies. The mode of transmission is not clear; both contamination of wounds from environmental reservoirs, such as bio films on aquatic vegetation [11] and infection through the bite of insect vectors [12-14] have been discussed.

Until recently, surgery has been the only WHO recommended treatment for BU [15-17]. Wide excision margins reaching into the healthy tissue are necessary to prevent recurrences [18] and often subsequent skin grafting is required. In most endemic areas access to surgery is very limited for the majority of BU patients. Moreover, the costs for treatment and prolonged hospital stays are often prohibitive. In 2004, WHO published provisional guidelines recommending treatment with a combination of rifampicin and streptomycin [19] based on results of a small randomised controlled clinical trial [20] and observational studies. While no antibiotic therapy has been formally proven effective in BU [17], there is evidence that treatment with a combination of rifampicin and streptomycin reduces recurrence rates and may help to avoid surgery or at least limit its extent [21]. More than 50% of BU cases are children below 15 years. Potential long-term side effects of streptomycin in this population

restrict the duration of the antibiotic treatment to eight weeks. If surgery is combined with antibiotic therapy, the aim is to use minimal surgery to excise necrotic tissue when antibiotics have arrested progress of the disease. For yet unknown reasons, a proportion of BU patients seem to be refractory for antibiotic treatment.

Histopathological hallmarks of progressing BU are a poor inflammatory response and growing regions of necrosis of the dermal and adipose tissue eventually leading to the collapse of the overlying epidermis (Figure 1; A to D). Clusters of extracellular, mycolactone-producing acid-fast bacilli are usually located within these necrotic areas (Figure 1; E and F) [18,22-24]. Granulomatous responses in the dermis and panniculus have been described in late stages of BU [18,22,25]. Observations both in cell culture and rodents experimentally infected with mycolactone producing and mycolactone-negative *M. ulcerans* strains indicate that infiltrating cells are killed due to the cytotoxic and apoptosis inducing activity of mycolactone [5,26,27]. While *M. ulcerans* may be captured by phagocytes during different stages of infection, it appears to persist only transiently inside these host cells [6,28]. After killing of the phagocytes, extracellular growth leads to the development of extracellular bacterial foci in areas of coagulating necrosis [18,27]. The aim of the present study was to analyse whether the local immunosuppression in Buruli ulcer lesions can be reversed by the combination treatment with rifampicin and streptomycin and if intralesional cellular immune responses complement antibiotic therapy.

Materials and Methods

Clinical specimens

Surgical specimens from five patients aged between six and 11 years with ulcerative lesions were obtained from the Amasaman Health Centre in Ghana and the Ayos district hospital in Cameroon (Table S1; supplementary material). Lesions were not older than three months and located at the lower leg (four patients) or arm (one patient). Patients had received the currently recommended standard treatment comprising surgical excision of lesions after pre-treatment with a combination of rifampicin and streptomycin (WHO, 2004). Although no further ulcer enlargement and reduction of oedema were observed, the responsible clinicians had decided to treat all five patients surgically to remove necrotic tissue and facilitate wound healing. After receiving informed consent from the guardians of the patients, surgical specimens were used for laboratory reconfirmation of clinical diagnosis and for detailed immunohistochemical analysis. All five specimens were positive for at least two of three diagnostic tests applied (Buruli ulcer - Diagnosis of *Mycobacterium ulcerans* disease, WHO, 2001), IS2404 PCR, microscopic detection of acid-fast bacilli and observation of characteristic histopathological changes. After receiving informed consent the immunologically non responding patient was tested for HIV positivity. Ethical approval for analysing patient specimens was obtained from the ethical review board of the Noguchi Memorial Institute for Medical Research and the National Ethics Committee of Cameroon.

Immunohistochemistry

Immediately after surgery, specimens with a volume of about 0.5 cm^3 were collected from different areas of the excised lesions to characterize the gradient of histopathological changes from necrotic areas to healthy appearing tissue at the excision margins (Figure S1; supplementary material). Tissue samples were fixed overnight in neutral buffered 4% paraformaldehyde, transferred to 70% ethanol, embedded in paraffin according to standard protocols and cut into 5 µm sections using a microtome. After deparaffinization sections were rehydrated through graded alcohols, endogenous peroxidase was blocked with 0.3% H_2O_2 for 20 min and unspecific binding prevented by incubating with blocking serum matching the

secondary antibody host (Table1). Antigen retrieval treatment was performed according to standard protocol (Dako®). After antigen retrieval antibodies (Table 1) were diluted in PBS containing 0.1% Tween-20 and slides incubated for 1 h at room temperature under rocking conditions. Afterwards sections were incubated for 30 min with a correspondent biotin-conjugated secondary antibody (Table 1) and for another 30 min with streptavidin-horseradish peroxidase conjugate (VECTASTAIN® ABC Kit, Vector Laboratories). Staining was performed using Vector® NovaRED™ and haematoxylin (counterstain). Slides were subsequently mounted with Eukitt® mounting medium.

Staining with Ziehl Neelsen (ZN) and Haematoxylin/ Eosin (HE) was performed on all collected tissue specimen. Staining for acid-fast bacteria was performed according to WHO standard protocol (WHO Diagnostic booklet). In brief, sections were deparaffinized and rehydrated followed by incubation with ZN carbolfuchsin for 30 min at RT. Subsequently slides were washed in cool tap water for 5-10 min and individually differentiated with acid-alcohol. Counterstain was completed with haematoxylin and slides were mounted with Eukitt® mounting medium.
Pictures taken with a Nikon optiphot-2 microscope were saved using analySIS® soft imaging system and processed with Adobe Photoshop® CS.

Results

Lack of a marked inflammatory response and abundant clusters of extracellular bacilli in one of five antibiotic treated patients

The excised lesion of one of the five antibiotic treated patients was almost devoid of cellular infiltrates. Only minor superficial vasculitis-associated infiltrates of the dermis consisting of lymphocytes and macrophages/ monocytes were found (Figure 2A). Extensive connective tissue necrosis and epidermal hyperplasia was observed (Figure 2A). The adipose regions displayed fat cell ghosts, extensive calcification and massive necrosis, associated with nearly complete absence of intact cell structures (Figure 2B; calcified areas stained purple). While the ulcerative centre of the lesion harboured numerous large clusters of rod-shaped extra-cellular acid fast bacilli (Figure 2C), margins contained only very few small bacterial microcolonies (not shown). A polyclonal antiserum, raised against *M. leprae* and highly cross-reactive with other mycobacteria, was used to stain *M. ulcerans* for confirmation of ZN staining results. Staining of common mycobacterial antigens with pAbLep antiserum and haematoxylin counterstain revealed an accumulation of mycobacteria (Figure 2D; red-brown) close to fat cell ghosts and necrotic calcified tissue (purple). In-between fat cell ghosts leukocytes exhibiting signs of defragmentation of nuclei and loss of cytoplasm were found only sporadically (Figure 2E). Taken together, features of this lesion resembled that of specimens from untreated patients (Figure 1) [18]. The patient was tested negative for HIV.

Highly organized inflammatory responses and intracellular bacterial material in four of five antibiotic-treated patients

In the other four patients massive cellular infiltration was observed. Three major types of mixed infiltrates, differing in cellular composition, architecture and localisation were found in all four specimens: (i) highly organized epithelioid granulomas of different size and state of differentiation, primarily located in deeper dermal tissue (Figure 3A); (ii) less organized diffuse infiltrates representing the most abundant type, present in all areas of the dermal connective and adipose tissue (Figure 3B); (iii) dense lymphocyte clusters in proximity to vessels, occasionally found in superficial connective tissue (Figure 3C). Cellular composition and

localisation of these structures along with the distribution of mycobacterial material are also schematically represented in Figure 3.

Histopathological characteristics of BU were still found in all four patients. These included psoriasiform and pseudoepitheliomatous epidermal hyperplasia and depigmentation (Figure 4A) with exceeding proliferation of keratinocytes (Figure 4B). Additionally, extensive necrosis of adipose tissue resulted in the appearance of fat cell ghosts (Figure 4C). Cellular infiltration was generally more profuse in vicinity to the necrotic centre of a lesion and declined towards the excision margins. Granulomas were composed of foamy histiocytes and Langhans' giant cells surrounded by lymphocytes and some plasma cells (Figure 4A). No central caseous necrosis was observed. In deeper adipose tissue dense infiltration with high proportions of macrophages and new blood vessel formation were observed (Figure 4C). Occasionally signs for calcification of deep dermal tissue were present (not shown). Areas of necrotic connective tissue were encircled by large accumulations of leukocytes (Figure 4D). Some leukocytes exhibiting apoptotic features resided inside necrotic regions (Figure 4D). The periphery of lesions revealed focal superficial eosinophilia (Figure 4E).

The distribution of mycobacterial material was assessed by ZN staining (Figure 5A - 5D) and immunostaining with pAbLep antiserum (Figure 5E - 5G). Both staining methods were found to be equally sensitive when compared in serial sections (Figure 5A and 5B vs. 5E and 5F). In all four patients mycobacterial material was predominantly found inside macrophages, although some was still located extra-cellularly; both extra- and intracellular bacilli had lost their characteristic rod-shape appearance (Figure 5B and 5F). While the cytoplasm of macrophages frequently harboured numerous phagosomes containing mycobacterial material (Figure 5C), only a few Langhans' giant cells with vacuoles containing ZN and serologically stainable material were located within granulomas (Figure 5D and 5G, respectively). The highest burden of mycobacterial material was found in deep dermal regions (Figure 5A and 5E) with a declining gradient from ulcerative areas towards the excision margins. Accumulations of mycobacterial material were primarily observed in areas of mixed cellular infiltration, but only very rarely in granulomas. Additionally, few residues of microcolonies were present in the upper dermal connective tissue or close to the epidermal basal layer (not shown).

Architecture of granulomas

A panel of antibodies specific for leukocyte markers (Table 1) were used for immunohistochemical characterisation of infiltrates. Serial sections revealed that the outer layer of granulomas was mainly composed of $CD3^+$ T lymphocytes (Figure 6A) with $CD4^+$ T cells (Figure 6B) always outnumbering $CD8^+$ T cells (Figure 6C). These lymphocyte belts were interspersed with $S100^+$ dermal dendrocytes (dDCs) (Figure 6D). Occasionally dDCs were present in the centre of granulomas (not shown). Focal clusters of $CD20^+$ B cells appeared at the outer margins of the T lymphocyte layer (Figure 6E). Cytoplasmic $CD68^+$ antigen-presenting cells (APCs) in particular Langhans' giant cells (Figure $6F_{insert}$) and epithelioid macrophages formed the centre of granulomas (Figure 6F). Staining of the membrane protein CD14 confirmed the macrophage/monocyte origin of giant cells (Figure 6G, arrow). Furthermore, large amounts of soluble CD14 were observed within the belt of T lymphocytes (Figure 6G, arrowhead). A great proportion of lymphocytes turned out to be activated as evidenced by their $CD45RO^+$ phenotype (Figure 6H). Variable numbers of $Ki67^+$ (proliferating) cells (Figure 6I) presented a lymphocyte phenotype (Figure $6I_{insert}$). Neither $elastase^+$ polymorphonuclear neutrophilic leucocytes (PMNL) nor $CD56^+$ natural killer (NK) cells could be detected in granuloma formations.

Cellular composition of diffuse mixed infiltrates

In contrast to granulomas, regions of diffuse cellular infiltration contained sparsely distributed $elastase^+$ PMNL (Figure 7A) with sporadic focal clusters near ulcerative and necrotic areas (not shown). $CD56^+$ NK cells showed a similar distribution with even lower cell counts (not shown). Foci of PMNL and NK cells with signs of apoptosis were located within necrotic tissue (Figure $7A_{insert}$ and 7B, respectively). $CD3^+$ T lymphocyte (Figure 7C) and foamy histiocytes (not shown) were the most prominent cell types of mixed infiltrates in the dermal connective tissue. The $CD4^+/CD8^+$ cell ratio varied widely between different regions of a lesion, but usually $CD8^+$ cells were more abundant than $CD4^+$ cells (not shown). Small clusters of $CD20^+$ B lymphocytes were scattered within infiltrates (Figure 7D). Counts of $CD14^+$ macrophages/monocytes were particularly high in adipose tissue and around necrotic areas (Figure 7E). Additionally, vast amounts of sCD14 shed from those cells were revealed (Figure $7E_{insert}$). Dendritic cells could be detected in both epidermis and dermis. The frequency of epidermal $CD1a^+$ Langerhans cells was strongly elevated

(Figure 7F). $S100^+$ dDC were distributed throughout diffuse mixed infiltrates with increasing density towards the margins of necrotic areas (Figure 7G). Remarkably, dendritic cell appendices reached into the damaged tissue (Figure 7H). Similar to the findings in granulomas, a large proportion of lymphocytes stained positive for the activation marker CD45RO (Figure 7I) and proliferating $Ki67^+$ cells were scattered throughout the infiltrate (Figure 7J).

Aggregates of lymphocytes resembling follicular structures

Adjacent to lymphatics or blood-vessels a third type of infiltration was found, i.e. follicle-like structures with dense aggregations of lymphocytes (Figure 8A). $CD20^+$ B lymphocytes were the dominating cell type (Figure 8B) followed by $CD3^+$ T lymphocytes (Figure 8C). Here $CD4^+$ T cells (Figure 8D) were more frequent than $CD8^+$ T cells (Figure 8E). Additionally, few $CD68^+$ APCs (Figure 8F) and $S100^+$ dDCs (Figure 8H) were scattered throughout the aggregate. Similar to the other two infiltration types, a large proportion of lymphocytes stained positive for the activation marker CD45RO (Figure 8G). Single proliferating $Ki67^+$ cells were evenly distributed (Figure 8I) and sporadically hyper-proliferative clusters were encountered (Figure $8I_{insert}$).

Discussion

It has been demonstrated recently that treatment with a combination of rifampicin and streptomycin inhibits the growth of *M. ulcerans* in pre-ulcerative BU lesions [19]. Furthermore, observational studies [21] indicate that at least in some of the BU patients antibiotic therapy may reduce the extent of or even circumvent the indication for surgery. Here we present results of a detailed immunohistological analysis of ulcerative BU lesions from patients treated with rifampicin/streptomycin prior to surgery. Findings were compared to those obtained from lesions of patients that have been treated merely with wide-ranging surgical excision (Figure 1) according to former WHO treatment guidelines [1,18]. To our knowledge this is the first detailed immunohistological description of the effect of antibiotic treatment on BU lesions.

Presentation of the affected tissue of four of the five antibiotic treated patients was strikingly different from that of untreated patients, in that immense leukocyte infiltrates and the formation of new vessels were observed. These findings reconfirm results of Etuaful et al., who observed an induction of chronic inflammation and granulomas in pre-ulcerative lesions by antibiotic treatment [19]. Our detailed analysis regarding composition and architecture of cellular infiltrates is indicative for the development of highly organized tertiary lymphoid tissue. Islands of infiltration and scattered granulomas may also develop in untreated late stage lesions [18,22,25]. However, infiltration in the patients at hand was much more substantial, resembling histopathological patterns observed in conditions such as dermatoborreliosis, where lymphoid neogenesis is reported [29]. We found three different types of infiltration in all four responding patients (Figure 3): granulomas, diffuse mixed infiltrates and dense lymphocyte aggregations in the vicinity to vessels. We assume that this clear structural differentiation reflects a range of different functional activities required for complete clearance of infection and resorption of necrotic tissue.

In regions of diffuse infiltration, in particular close to areas of necrosis, outstandingly large amounts of both membrane-bound and soluble CD14 were found. Consistent with this, it has been reported that the development of highly organized structures, such as granulomas, is not required for the resorption of destroyed tissue [30]. CD14 mediated clearance of necrotic tissue is usually not associated with an increased expression of inflammatory cytokines [30], which is in line with the observed lack of

major inflammatory symptoms in the enrolled patients. Necrotic regions were surrounded by a substantial quantity of dDCs and their appendices reached into the damaged tissue, indicating enhanced antigen uptake and presentation. Moreover, elevated numbers of Langerhans cells were distributed throughout the epidermis, like it has been described for tuberculoid and borderline leprosy [31]. A large proportion of lymphocytes were expressing activation and proliferation markers. Taken together, these observations reveal that adaptive immune responses are taking place autonomously.

While in human tuberculosis granulomas develop a central necrotic core [32], this was not observed in the lesions of the antibiotic treated BU patients. Here, the centre was mainly formed of foamy histiocytes and Langhans' giant cells, like reported for leprosy [33], and mycobacterial material was only infrequently detected. In leprosy, cells of the lymphocyte belt are less tightly packed [34] than in tuberculosis [35], a feature we also observed in BU lesions. Thus, BU granulomas may function primarily as a place for antigen presentation and adaptive immune response rather than for sequestration of the mycobacteria.

In diffuse infiltrates the CD8/CD4 T lymphocyte ratio was higher than in granulomas. Otherwise, the cellular composition of both types of infiltration was largely the same. Small islets of Langhans' giant cells inside the diffuse infiltrates support the hypothesis that granulomas represent a more advanced state of the initially unorganized infiltrations. In contrast, the dense lymphocyte aggregations we observed in vicinity to vessels were of a markedly different cellular composition. Here B lymphocytes represented the most dominant cell type in contrast to granulomas or unorganized infiltrates, where small B cell clusters were distributed more sparsely. B and T lymphocytes were packed in a notably dense manner, with interspersed dDCs and APCs and no central core, a composition characteristic also for secondary lymphoid organs. Similar structures have also been reported by Ulrichs et al in lung tissue from tuberculosis patients [35]. Many lymphocytes were in the activated state as demonstrated by CD45RO staining and a substantial proportion displayed the proliferation marker Ki67. It has been suggested that these cell aggregations represent active centres, which orchestrate the local host defence [35]. It is not clear, which factors play a crucial role in the development of ectopic lymphoid tissue induced via antibiotic treatment. Plasmacytoid and myeloid dendritic cells present in BU lesions prior to antibiotic treatment [36] may play an important role in this

process. Lymphangiogenesis and participation of lymphoid tissue-inducer cells may represent hallmarks in the process of lymphoid neogenesis [29]. Although tertiary lymphoid organs seem to develop in infectious diseases to sequester pathogens, this process is often accompanied by tissue damage [29]. Anecdotal reports on the emergence of new ulcerations in the course of antibiotic treatment of BU, probably at sites containing unrecognized infection foci, may be a hint into this direction. It remains to be analysed whether in some cases antibiotic treatment may even accelerate the progression of early plaques and oedemas to ulcerative lesions.

In animal infection models neither wild type nor mycolactone negative *M. ulcerans* strains were strong neutrophilic attractants [6], although other studies reported substantial neutrophilic infiltrates in early stage infections [28]. Infiltrates in patients treated with antibiotics contained only very low levels of neutrophilic leukocytes. In contrast, large numbers of apoptotic cells positive for the neutrophilic leukocyte marker elastase and the NK cell marker CD56 were present within necrotic regions. Findings with an experimental *M. ulcerans* mouse infection model [27] indicate that these apoptotic cells represent the residues of an early acute defence line. Our data show for the first time that cells of this early defence are destroyed together with the surrounding tissue, if a substantial infection focus develops. It is most likely that the generalized cytotoxic activity of mycolactone is the all-dominant factor.

Lesions of all four responders still harboured mycobacterial material as revealed by ZN and immunohistochemical staining. However microcolonies consisting of extracellular [9,22] or intracellular [37] rod-shaped mycobacterial cells were no longer found. Stainable mycobacterial material had lost the characteristic shape and was primarily located inside mononuclear phagocytes, but was also present in small extracellular foci. These data indicate a killing or debilitation of the bacteria in the course of antibiotic treatment. Moreover, antigen uptake and presentation via macrophages and dendritic cells may be enhanced due to lower levels of mycolactone. While bacterial residues were abundant in regions of diffuse infiltration of the deeper dermis and in adipose tissue, granulomas were largely devoid of it. Only single Langhans' giant cells contained vacuoles filled with stainable residues of the mycobacterial cells. A similar pattern may be found in active pulmonary tuberculosis [35,38]. Again, these findings support the theory that granulomas serve as a place for antigen encounter.

The density of stainable mycobacterial material increased from the outer margins of lesions towards the ulcerative centre, which is consistent with the distribution of mycobacterial DNA in untreated lesions [18,39]. Moreover, our results demonstrate that after antibiotic treatment the mycobacteria are directly taken up by phagocytes at the original focus of infection without granuloma formation. *M. ulcerans* is able to destroy phagocytes after transient intracellular growth via the release of mycolactone [6,28]. We think that the stainable intracellular mycobacterial material represents the remains of phagocytosed dead bacilli or of cells severely impaired in mycolactone production due to the antibiotic treatment. In general it is most likely, that a pronounced reduction of the concentration of mycolactone in the lesion is a prerequisite for the observed reversal of the local immunosuppression. Due to the lack of quantitative detection methods for mycolactone, for the time being this aspect cannot be studied.

The BU lesion of one antibiotic-treated patient displayed histopathological features similar to active lesions of patients not receiving antibiotic treatment. Only minor leukocyte infiltration was found and huge clusters of extracellular mycobacteria were located beneath the ulcerative centre. Observational studies indicate that in a certain proportion of patients, rifampicin/streptomycin treatment has no clinical curative effect. Acquired or inherited host factors, like deficiencies in the IFN-γ and IL-12 pathways for macrophage activation [40,41], may play a role. Alternatively, antibiotic resistance of certain lineages of *M. ulcerans* could be responsible for this lack of response. Therefore major efforts should be made to generate *M. ulcerans* isolates from these patients for susceptibility testing. Another factor to be considered is poor patient compliance. Rifampicin is administered once per day orally and the intake of tablets may not be monitored. Furthermore, outdated drugs and poorly prepared solutions can be possible reasons for treatment failure and clearly need to be taken into consideration.

In summary, we conclude that treatment of BU with rifampicin/streptomycin is accompanied by a reversion of the local immunosuppression. The synergistic anti-mycobacterial action of antibiotics and immune defence mechanisms may be required to clear the infection efficiently. In view of these results, it may be suitable to replace streptomycin already after a few weeks by a bacteriostatic antibiotic. Recently published data of our group indicate that not everyone exposed to *M. ulcerans* develops clinical disease [42]. After exposure a race between front line

immune responses and mycobacterial multiplication seems to decide whether a chronic infection focus is established or not. Once a bacterial cluster is large enough to develop a cytocidal cloud of mycolactone around itself, necrotic areas with only minor infiltration develop [18,22-25,43], which is in stark contrast to the vigorous immune responses we describe in the present study. We infer that a complex and highly organized cellular immune response is crucial for elimination of a chronic *M. ulcerans* infection. Efficient triggering of local immune responses may thus represent a suitable auxiliary marker for the efficacy of alternative treatment strategies.

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Nonstandard abbreviations

APC	antigen presenting cell
BU	Buruli ulcer
dDC	dermal dendritic cell
HE	haematoxylin/ eosin
pAbLep	polyclonal anti-leprae antibody
PMNL	polymorphonuclear neutrophilic leukocytes
ZN	Ziehl Neelsen

Figures

Table1. Antibodies used for immunohistochemistry

target antigen	stained cell type(s)	host (clone)	working dilution	retrieval method	source
CD1a	Langerhans cells	mouse (O10)	prediluted	Citrat	Beckman Coulter
CD3	T lymphocytes	rabbit	1/100	Citrat	Dako
CD4	T helper lymphocytes	mouse (1F6)	1/100	EDTA	Novocastra
CD8	T cytotoxic lymphocytes	mouse (4B11)	1/100	EDTA	Novocastra
CD14	Phagocytes	mouse (7)	1/50	Citrat	Novocastra
CD20	B lymphocytes	mouse (7D1)	1/100	Citrat	Novocastra
CD45RO	Activated lymphocytes	mouse (UCHL1)	1/100	none	Dako
CD56	Natural Killer cells	mouse (SPM489)	1/100	Citrat	Lab Vision
CD68	Antigen presenting cells	mouse (KP1)	1/50	Trypsin	Dako
elastase	Neutrophilic leucocytes	mouse (NP57)	1/50	none	Dako
Ki67	Proliferating cells	rabbit	1/50	Citrat	Dako
S100	Dermal dendrocytes	rabbit	1/400	Trypsin	Dako
mycobacterial antigens	Mycobacteria	pAbLep mouse antiserum	1/1000	Citrat	Leprosy Research Support
biotinylated anti-rabbit IgG	Secondary antibody	goat	1/200	-	Vector
biotinylated anti-mouse IgG	secondary antibody	horse (rat adsorbed)	1/200	-	Vector

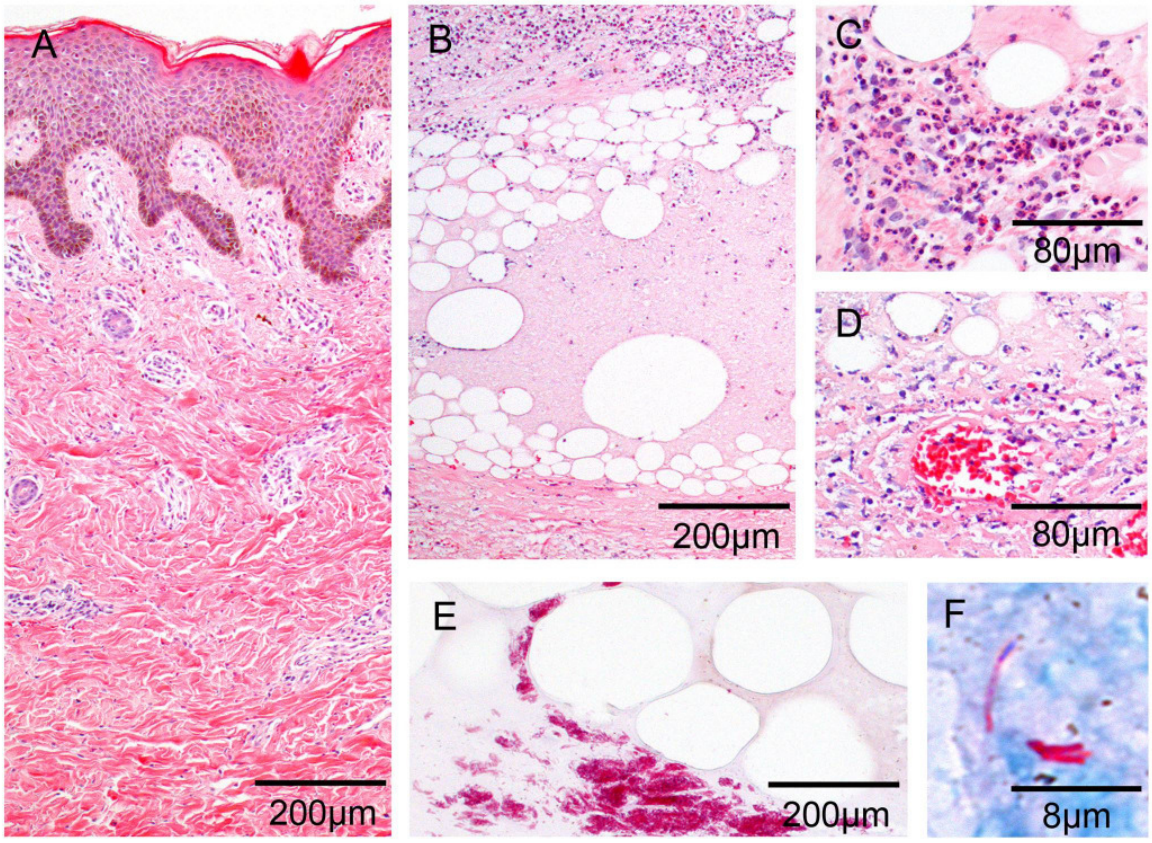


Figure1. Histopathological characteristics associated with untreated Buruli ulcer lesions

Histological sections of specimen from untreated Buruli ulcer lesions stained with HE (A to D) and ZN (E, F), respectively. Photographs are taken at magnification x40 (A, B, E), x100 (C, D) or x1000 (F). (A) Vasculitis associated minor leukocyte infiltration around vessels with intact dermal connective tissue and epidermal hyperplasia. (B) Extensive areas of necrosis in the deeper dermis and large fat cell ghosts with slight leukocyte infiltrates. (C) Slight cellular infiltration mainly composed of PMNL. (D) Ongoing necrotic/ apoptotic processes surrounding a focus of mycobacterial microcolonies. (E) Typical clusters of extracellular bacteria between adipose cell ghosts. (F) Sometimes also single bacilli can be spotted inside necrotic regions.

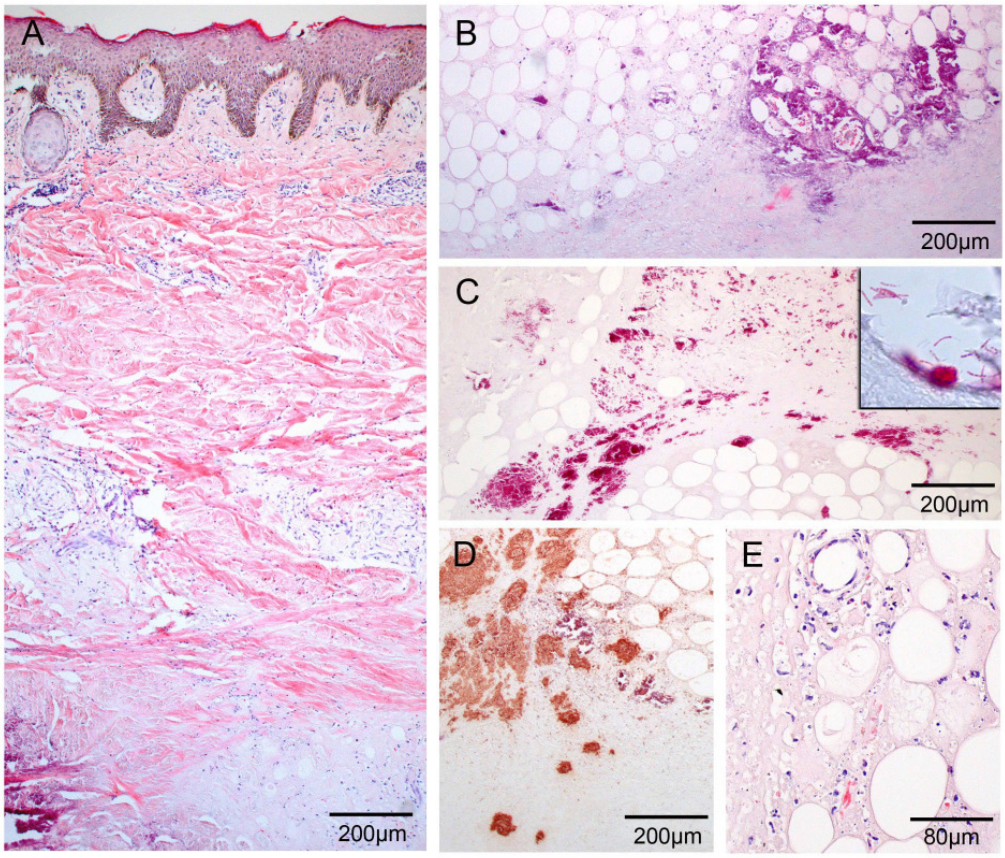


Figure2. One patient exhibits strong histopathological signs for a progressive Buruli ulcer and large mycobacterial clumps

Histological sections of the non-responding patient stained with HE (A, B, D) and ZN (C) or polyclonal anti-leprae antibody (pAbLep; E). Magnification x40 (A, B, C, E) and x100 (D). (A) Tissue shows typical signs of advanced Buruli ulcer as deep dermal necrosis, calcification or epidermal hyperplasia. Leucocytes are found in rare cases around vessels and hardly ever between fat ghosts. (B) Adipose tissue and its surroundings are highly necrotic and happen to accumulate fat ghosts and calcification. (C) The centre of the necrotic lesion harbours tremendous clumps of rod-shaped mycobacteria. (D) Staining with pAbLep demonstrates the focal clusters of live bacteria in the necrotic core. (E) If cellular infiltration occurs in small amounts within some parts of the intradermal adipose tissue cells display apoptotic features.

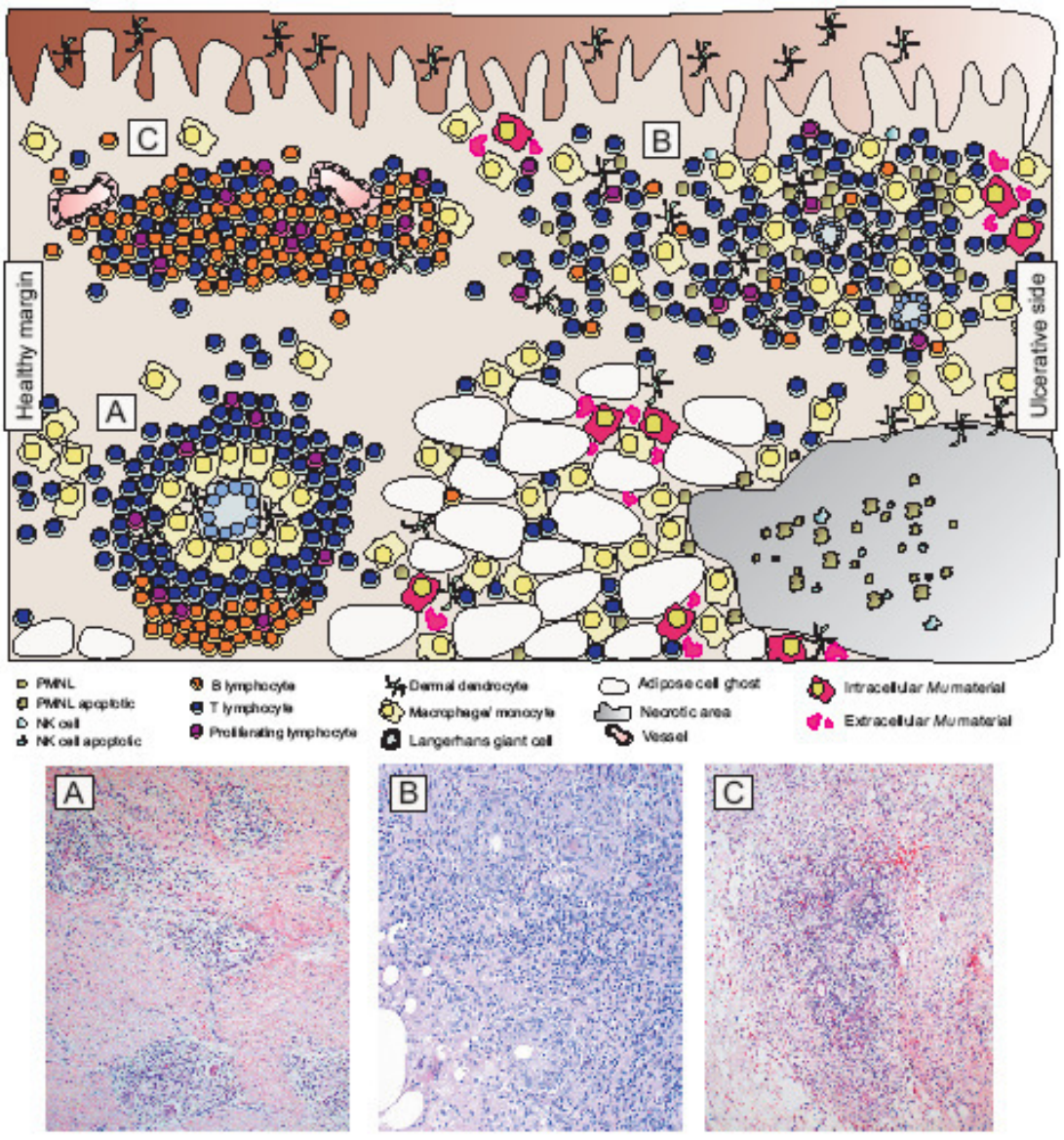


Figure3. Three major types of cellular infiltration can be distinguished in antibiotic treated BU patients

Upper part: Schematic overview of cellular infiltration patterns and distribution of mycobacterial material. Lower part: Three types of cellular infiltration are documented with HE. (A) Granuloma formation in the connective tissue; magnification x40. (B) Diffuse heterogeneous cellular infiltration of the connective and adipose tissue; magnification x100. (C) Follicle-like lymphocyte focus adjacent to vessels; magnification x40.

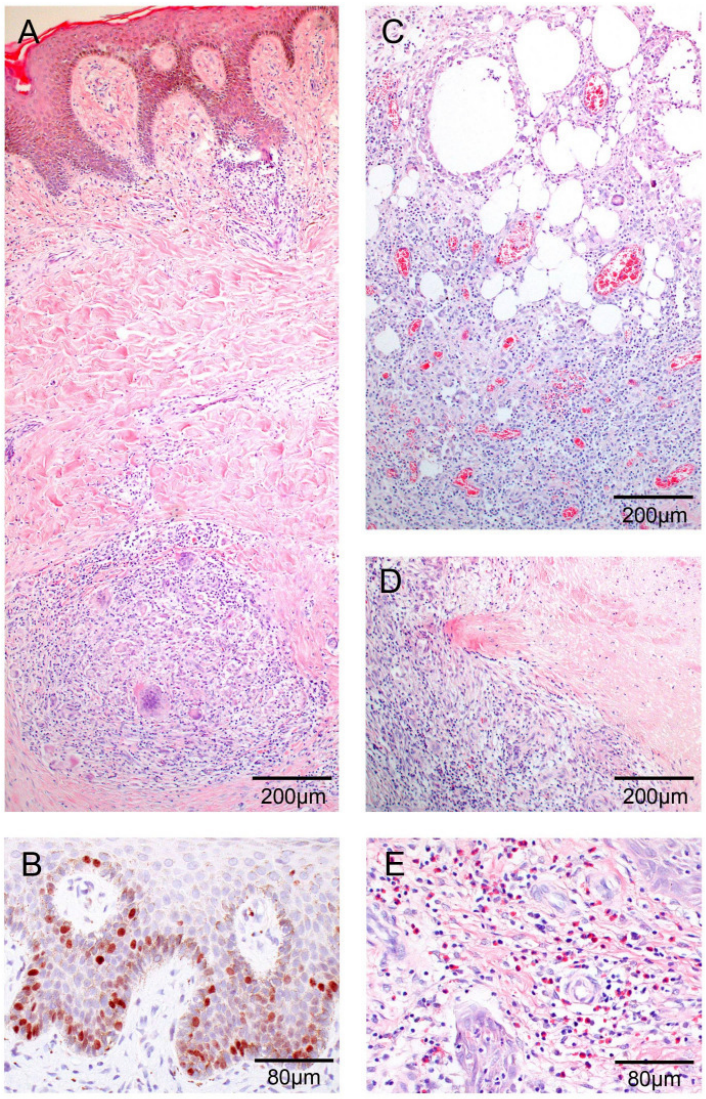


Figure4. Histopathology of four patients in response to rifampicin/ streptomycin bi-therapy

Histological sections representative for four patients stained with HE (A, C, D, E) and polyclonal antibody against proliferation marker Ki67 (B). Magnification x40 (A, C, E) and x100 (B, E). (A) Psoriatic epidermal hyperplasia typically seen in Buruli. Diffuse mixed cellular infiltrates in upper and granuloma formation with Langhans' giant cells lymphocytes in deeper dermis. (B) Ki67 staining reveals elevated proliferation levels of keratinocytes in epidermal basal layer. (C) Cell ghosts of the adipose tissue characteristic for Buruli infection. Massive mixed cellular infiltrates between fat ghosts mainly consisting of macrophages/ monocytes and formation of new blood vessels. (D) Necrotic area in deep tissue encircled by extensive cellular infiltrates. (E) Focal eosinophilia found at margins of the excised area distant to ulcerative centre.

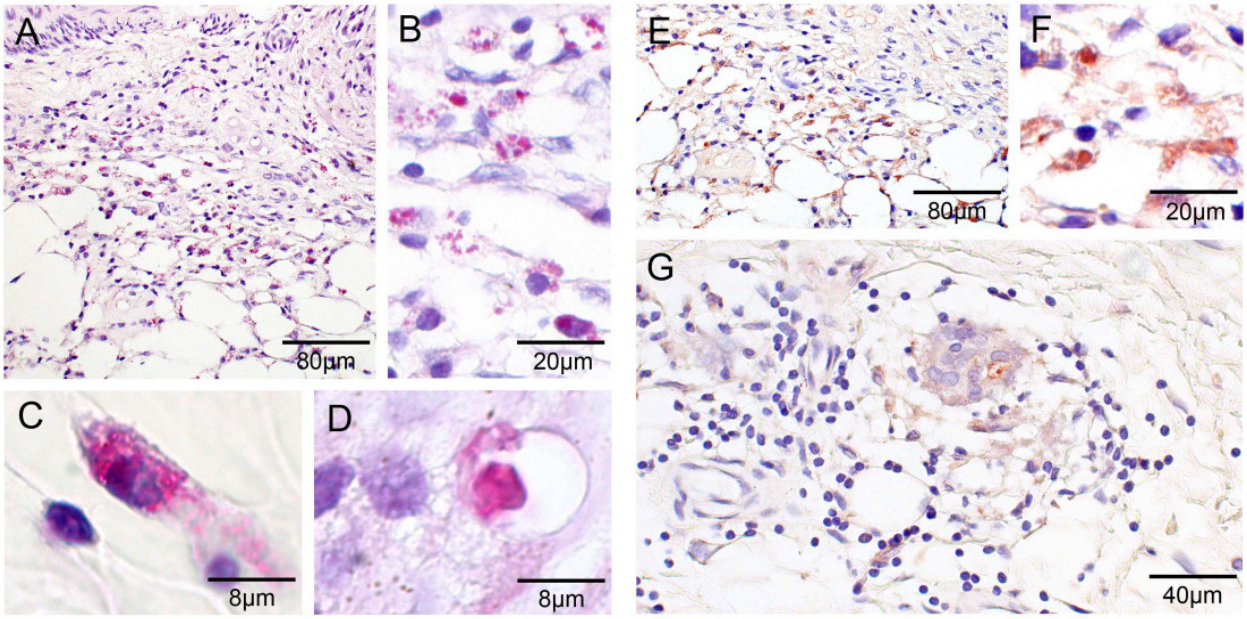


Figure5. Bacterial load and distribution in four patients

Histological sections representative for four patients stained with ZN (A to D) and polyclonal anti-leprae antibody (pAbLep; E to G) to demonstrate distribution of mycobacterial material in the excised lesion. Counterstain was performed with haematoxylin and pictures taken at magnifications of x100 (A, E), x200 (G), x400 (B, F) and x1000 (C, D). (A, E) Serial sections demonstrate equal detection sensitivity with ZN and pAbLep. Higher amounts of bacterial material near the ulcerative edges with macrophages being the most prevalent leucocytes. (B, F) Close-up of A and E, respectively. Foci of rounded bacteria located extra- and intracellular between fat ghosts. (C) Macrophage presenting with mycobacterial material within phagosomal vesicles. (D) Mycobacterial residues appear in rare cases inside Langhans' giant cell phagosomal vacuoles. (G) Small granuloma with giant cell formation and traces of mycobacteria inside a phagosome.

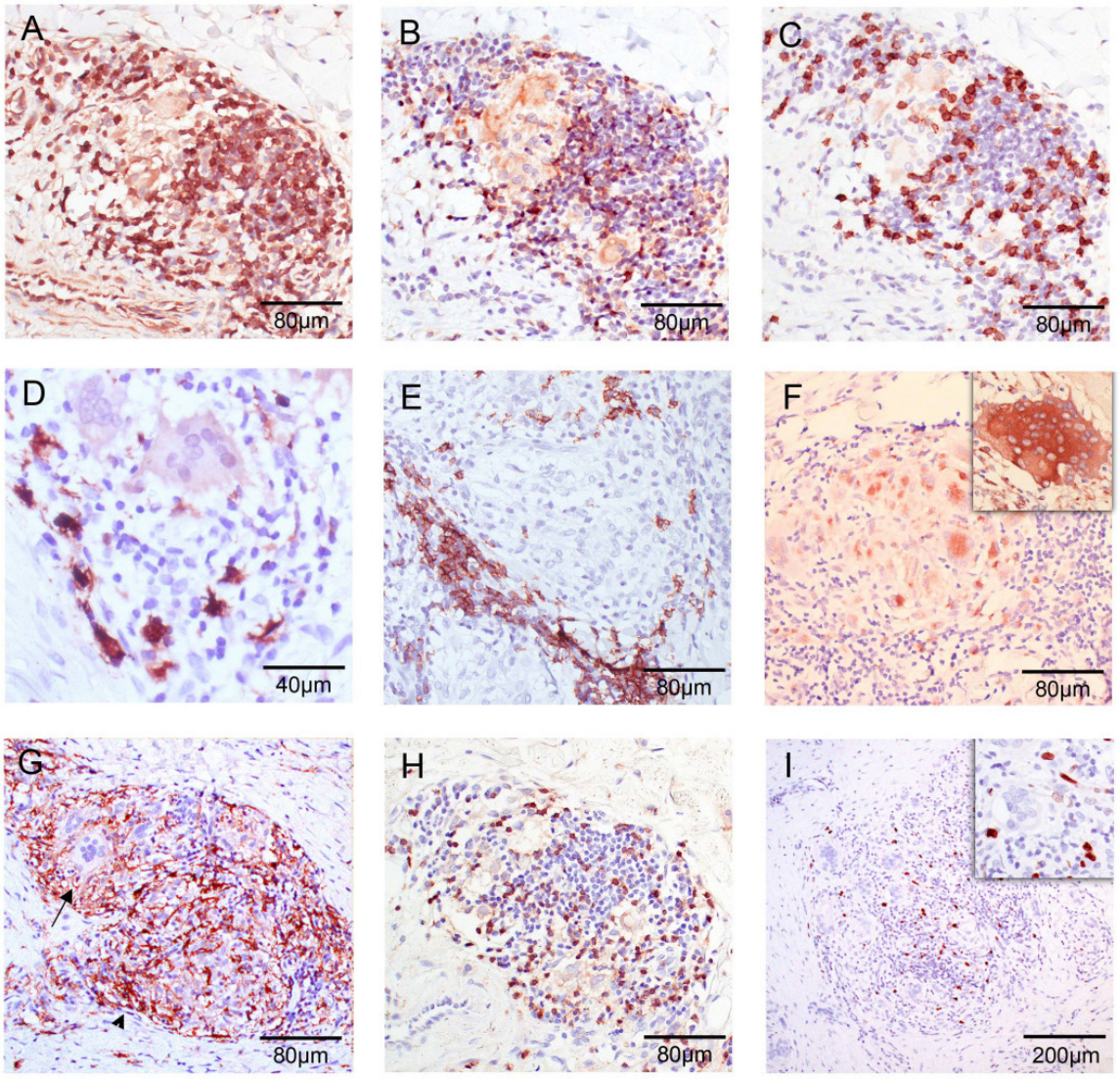


Figure6. Detailed organization of granulomas

Histological serial sections representative for four patients were stained with antibodies against different cellular surface or cytoplasmic markers (counterstain haematoxylin). Magnification x40 (I), x100 (A, B, C, E, F, G, H, I_{insert}), x200 (D) and x400 (F_{insert}). (A, B, C) Staining with CD3, CD4 and CD8, respectively, reveals a belt of helper as well as cytotoxic T lymphocytes surrounding the APC core. (D) $S100^+$ dermal dendrocytes (dDC) spread among T lymphocytes in the outer layer of a granuloma. (E) Focus of $CD20^+$ B lymphocytes at the border of a granuloma. (F) $CD68^+$ APC in the centre of a representative granuloma; insert shows large Langhans' giant cell. (G) Remarkable large amounts of membrane bound (arrow) and soluble (arrowhead) CD14 can be observed. (H) Distribution of activated $CD45RO^+$ lymphocytes. (I) Proliferating $Ki67^+$ cells indicate the active status of granulomas.

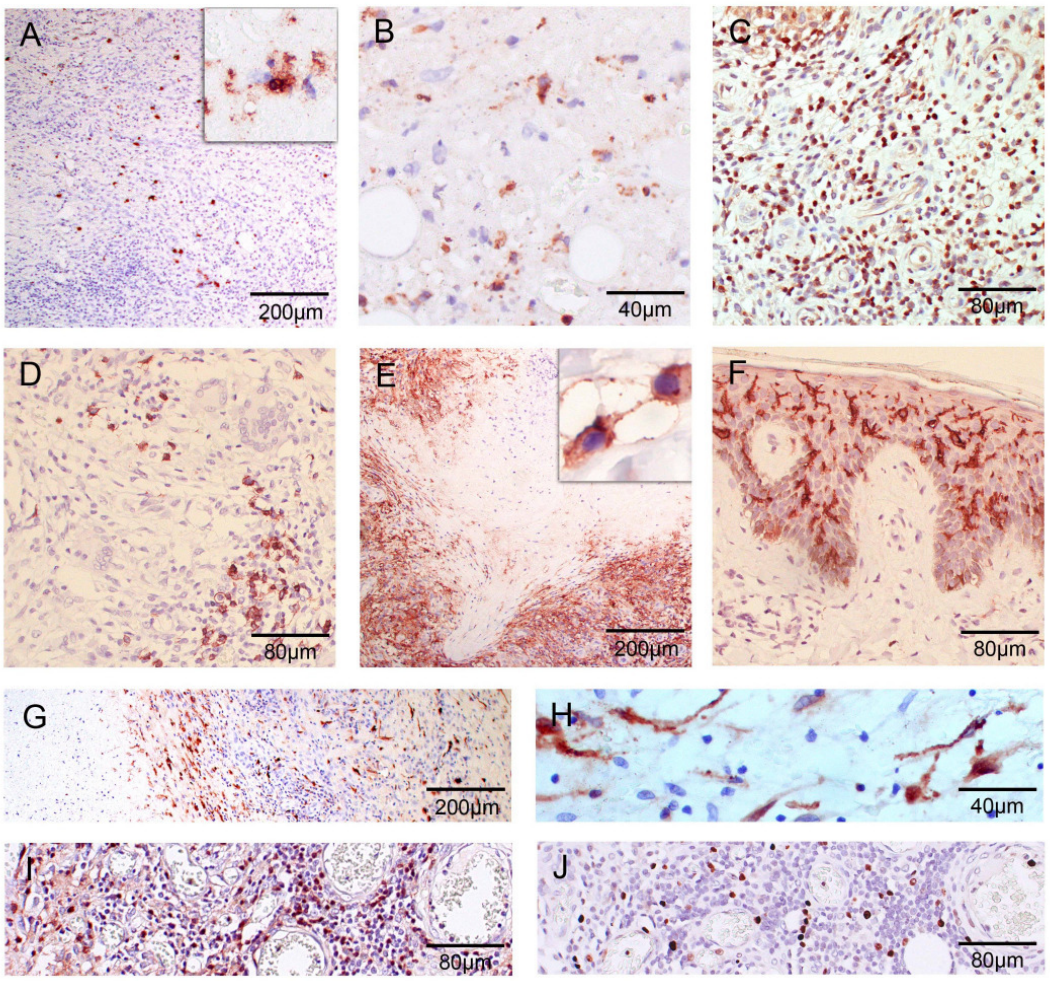


Figure7. Composite of mixed cellular infiltrates

Histological sections representative for four patients stained with antibodies against different cellular markers (counterstain haematoxylin). Magnifications at x40 (A, E, G), x100 (C, D, F, I, J), x200 (A_{insert}, B, H) or x1000 (E_{insert}). (A) Only few scattered PMNL staining positive for Elastase were found within cellular infiltrates. (A_{insert}, B) Neutrophilic and NK cell ($CD56^+$) foci inside necrotic areas display signs of advanced apoptosis. (C) Lymphocytes mainly expose a $CD3^+$ phenotype. (D) Small focal $CD20^+$ lymphocyte spots are scattered through infiltrates. (E) Staining against CD14 illustrates large numbers of histiocytes enclosing necrotic tissue shedding massive amounts of sCD14 (insert). (F) Levels of epidermal $CD1a^+$ Langerhans cells are remarkably elevated compared to healthy skin. (G) Aggregation of $S100^+$ dDCs near necrotic spots. (H) Elongated cellular appendices of dDCs reach into the necrotic tissue. (I) Large numbers of lymphocytes are $CD45RO^+$. (J) Same area as in (I). Proliferating lymphocytes are highly $Ki67^+$.

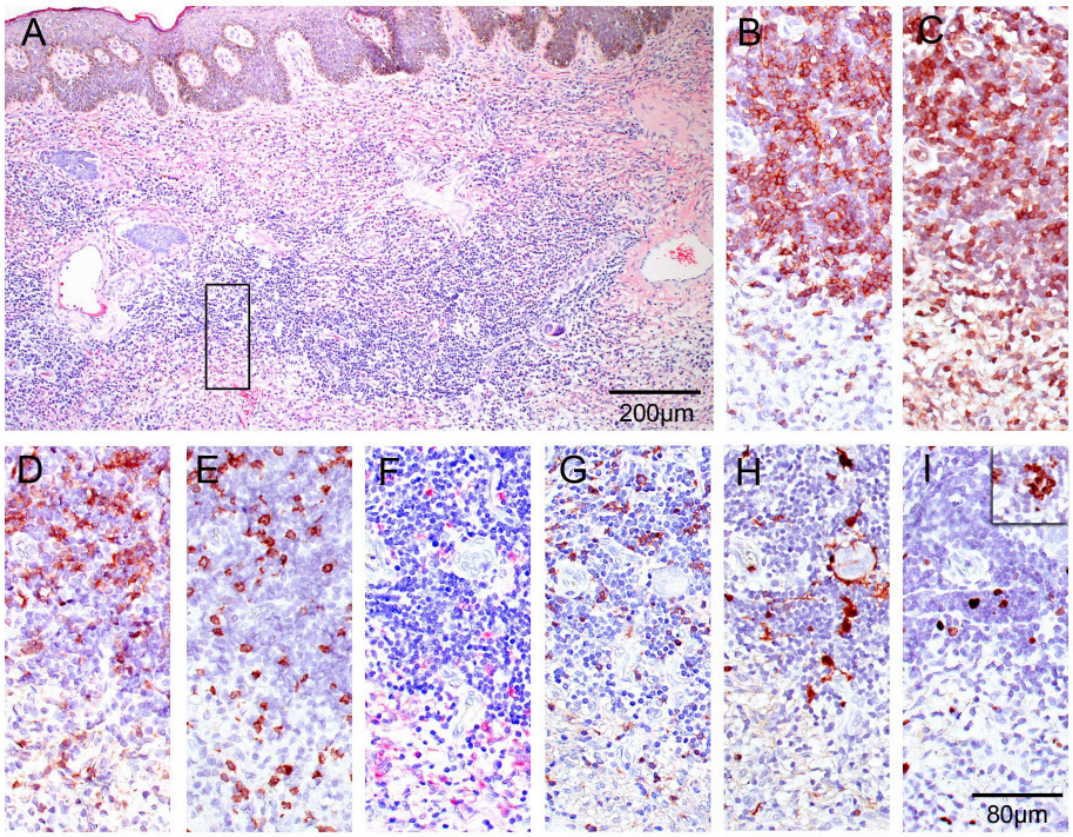
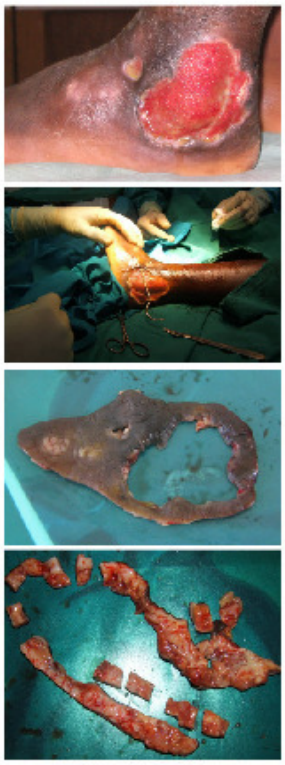


Figure8. Follicle-like lymphocyte foci reveal organized cellular formation

Serial sections of a representative lymphocyte focus stained with HE (A; magnification x40) or with antibodies against different cellular markers and haematoxylin as counterstain (B to I; magnification x100). (A) Large aggregate of lymphocytes in the upper dermal layer between two venules. Square marks area of magnification chosen for pictures B to I. (B) CD20+ B lymphocytes build the most abundant cell subset. (C, D, E) Staining against CD3, CD4 and CD8, respectively, exposes also high loads of T lymphocytes with T helper clearly being more prevalent than cytotoxic T cells. (F) APCs (CD68+) are scattered throughout the entire structure. (G) Activated lymphocytes appear positive for CD45RO staining. (H) S100+ dermal dendrocytes (dDC) lie in between lymphocytes. (I) Ki67 staining demonstrates certain proliferation of cells and occasional appearance of discrete spots of hyperproliferation (insert).

Supporting information

FigureS1. Sampling of tissue for immunohistochemistry



TableS1. Main data of enrolled patients

Patient	age	sex	location of lesion
1	6 years	m	right leg (above ankle)
2	7 years	m	right leg (backside)
3	7 years	m	left leg (around ankle)
4	8 years	m	right arm (ellbow)
5	11 years	f	right leg (thigh)

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CHAPTER 5

Phagocytosis of Mycobacterium Ulcerans in the Course of Rifampicin and Streptomycin Chemotherapy in Buruli Ulcer Lesions

D Schütte *, A Um-Boock †, G Pluschke *

* Swiss Tropical Institute, Molecular Immunology, Basel, Switzerland

† Leprosy Relief Emmaus-Switzerland (ALES) Cameroon, Yaounde, Cameroon

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Abstract

Background: Infection with *Mycobacterium ulcerans* involves devastating skin disease called Buruli ulcer (BU). Currently, dual therapy with rifampicin and streptomycin (R/S) for eight weeks as well as surgery are the standard treatments.

Objectives: To elucidate the processes taking place in Buruli ulcer lesions in the course of chemotherapy we performed an in-depth histological analysis of lesions after four weeks of rifampicin and streptomycin (R/S) treatment. Results are compared to findings in untreated and eight weeks treated lesions, respectively.

Patients/Methods: Tissue specimens were collected from patients without, after four and eight weeks of R/S treatment, respectively. Main features evaluated were local immune responses, histopathological alterations and bacterial distribution.

Results: After four weeks of R/S treatment we observed a large proportion of mycobacteria inside macrophages, occasionally forming globus-like aggregations. While distinct bands of inflammatory leukocytes surrounded the necrotic core in an ulcer and early granuloma formation was apparent in the healthy appearing margins, acute cellular infiltration covering the whole lesion had developed in a nodular lesion. In contrast, ulcerative lesions after eight weeks of chemotherapy show intra- and extracellular bacterial debris as well as the extensive presence of chronic infiltrates forming huge granulomas.

Conclusions: R/S treatment of BU results in a rapid onset of local cellular immune responses associated with phagocytosis of the extracellular *M. ulcerans*. This might be related to declining levels of the macrolide toxin mycolactone in the tissue thus leading to an enhanced chemotherapy-induced clearance of the infection.

Introduction

Buruli ulcer (BU) is a chronic infectious skin disease caused by *Mycobacterium ulcerans* which may produce pronounced deformities and disabilities when left untreated. *M. ulcerans* causes necrotizing lesions due to the release of a diffusible macrolide toxin with cytotoxic properties named mycolactone [1-3]. Although it can affect humans of any age group, a large proportion of patients are children below the age of 15 [4]. There are increasing case-numbers mainly in rural areas of Western Africa associated with swamps and slow flowing or stagnant water bodies [5-7], yet the mode(s) of transmission along with the reservoir(s) of this disease remain unknown.

Initially BU appears as a mobile subcutaneous nodule, a papule or a raised plaque. These develop either into slowly enlarging necrotic ulcers, or rapidly progressing oedema sometimes affecting a whole limb. The initial prolonged stages of BU lesions can be almost painless and patients do not show signs of systemic infection such as fever or malaise [8]. At later stages, lesions may heal on their own accord yet are often accompanied by contracting scars and ankylosis [9].

Surgical treatment without extended excision into healthy appearing surrounding tissue is associated with relapse rates of up to 47% [10-12]. In addition, access to health care facilities is limited due to the remote nature of most BU endemic areas, and treatment is often both lengthy and expensive [9]. Since 2004, chemotherapy for eight weeks with a combination of rifampicin and streptomycin is recommended by WHO [13]. Small and/or nodular lesions seem to be curable with low relapse rates, but additional surgery and/or skin graft are often needed for larger ulcerative lesions [14,15]. Inhibition of cellular immune responses in the infected skin [16-18] seems to be a result of mycolactone production [1,3,19,20]. However, especially in late stage Buruli ulcer, local cellular infiltration and cytokine production may occur [21-24].

We recently described the development of ectopic lymphatic tissue in BU lesions after eight weeks of R/S treatment [25]. Microcolonies of extracellular rod-shaped bacilli were no longer observed and only focal, most often intracellular, mycobacterial residues were revealed. Furthermore, in 2005 Etuaful et al could demonstrate that four weeks of chemotherapy render both *in vivo* and *in vitro* cultures negative [26].

Here we present results of a histological analysis of skin specimens obtained from two patients (one nodule and one ulcer) who had been treated with R/S during four weeks. Histopathological pattern, cellular immune responses and localisation of acid fast bacilli (AFB) are compared to commonly observed features in untreated as well as eight weeks antibiotically treated lesions.

Materials and Methods

Study population and sampling

In total, four patients with clinical diagnosis of BU were recruited in Ayos, Cameroon. Two controls were included as representative cases for histopathological alterations in (i) untreated lesions and (ii) eight weeks antibiotically treated ulcers, respectively. The first control was a 45 year old woman with a nodule and a small ulcer on the left calf, both which were excised before the start of antibiotic therapy based on the decision made by the local surgeon. The second control was a seven year old boy with an ulcer on his left foot who underwent surgery in order to prepare the area for skin grafting after the full course of eight weeks R/S.

The third patient, a 13 year old boy, presented a nodule of about 3,5 cm in diameter near his right eye with oedematous swelling of the surrounding area. The fourth patient was a 14 year old boy with an early ulcer on his left arm right above the elbow. His lesion changed from nodule to ulcer three weeks before treatment was commenced and the ulceration was <2cm in diameter. Antibiotic treatment with daily administration of streptomycin (15 mg/kg i.m.) and rifampicin (10 mg/kg orally) was started immediately and, due to the critical location, surgery performed after (iii) four weeks. Afterwards, both patients completed the full eight weeks course of chemotherapy.

Clinical diagnosis was confirmed by Ziehl Neelsen (ZN) staining for acid fast bacilli, histopathology and/or standard IS2404 real-time PCR. Ethical clearance was obtained from the National Ethics Committee in Yaoundé, Cameroon.

Tissue samples were obtained as previously described 25, fixed in neutral buffered 4% paraformaldehyde, embedded in paraffin and sectioned using a HM 335 E rotary microtome (MICROM International GmbH).

Staining procedures and section analysis

Immunohistochemistry (IHC) was performed as described in a previous study 25. In brief, sections were deparaffinized and rehydrated, endogenous peroxidase was blocked with H2O2 and unspecific binding prevented by incubating with blocking sera. Subsequently slides were pre-treated with the adequate antigen retrieval method and incubated at room temperature with (i) monoclonal antibodies against CD1a (Beckman Coulter), CD56 (Lab Vision), neutrophilic elastase, CD45RO and

CD68 (all Dako), CD4, CD8, CD14 and CD20 (all Novocastra) or (ii) polyclonal antibodies against S100 and Ki67 (both Dako) and mycobacterial antigens (Colorado State University, CO, USA; pAbLep). Afterwards sections were incubated with the secondary biotinylated antibody and labelled with streptavidin horseradish peroxidase conjugate (both Vector Laboratories). Staining was performed by applying Vector NovaRed (Vector Laboratories) and haematoxylin (Sigma). Immunofluorescent labelling was achieved using secondary antibodies conjugated with Alexa 488 and 568 (Invitrogen), respectively, and 4',6-Diamidino-2-phenylindo (DAPI) counterstaining. Slides were mounted with Eukitt® mounting medium.

HE and ZN staining were performed according to standard protocols. Pictures were taken with a Leica® DM5000B microscope equipped with a Leica® DFC300FX® camera. Images were saved using Leica® Application Suite and processed with Adobe Photoshop® CS. Whole section analysis was performed at different magnifications of all samples taken and representative areas were chosen for figure preparation. All data about infiltrating cells relate to the necrotic lesions core and its immediate surroundings (max. 1 cm). The healthy appearing margins of most untreated ulcerative lesions contain cellular infiltrates. The same holds true for late pre-ulcerative lesions though at lower rates [17,22].

Results

Histopathological features of (i) untreated and (ii) eight weeks antibiotically treated Buruli ulcer lesions from representative areas of typical lesions

(i) The nodular lesion excised without R/S treatment showed typical histopathological features of an untreated nodule, i.e. a necrotic focus with fat cell ghosts (Fig. 1-A) and minor focal acute infiltration of the connective and adipose tissue (Fig. 1-B). Foamy Touton giant cells were found surrounding degrading adipose cells (Fig. 1-B; arrows). Furthermore, abundant bacterial clusters were located in the necrotic connective and adipose tissue (Fig. 1-C) and the cytotoxic activity of mycolactone reached beyond the mycobacterial load (Fig. 1-D). Bacterial clusters and even single scattered bacteria had no contact to intact leukocytes (Fig. 1-E).

In the same patient a further early ulcerative lesion, the more advanced stage of BU, was detected showing extensive necrosis with decomposing leukocytes and erythrocytes (Fig. 1-F). At the borders of the central necrotic area a more substantial acute infiltration as well as large haemorrhages were noted (Fig. 1-G). Compared to the nodule, the untreated ulcerative lesion revealed a very high bacterial load inside a huge necrotic compartment (Fig. 1-H). Some acute leukocyte infiltration, located at the circumference of the bacterial distribution (Fig. 1-I) was noticed being in close contact with extracellular mycobacteria and exhibited apoptotic signs such as nucleus defragmentation (Fig. 1-J).

(ii) In contrast, a patient who underwent eight weeks of R/S therapy showed diffuse mixed chronic leukocyte infiltrates in an early ulcer, which appeared to cover the necrotic area (Fig. 1-K) and fully developed granulomas were present towards the lesion margins (Fig. 1-L). Only ZN stainable bacterial debris was identified (Fig. 1-M), most of it located encased in macrophages (Fig. 1-N) which was confirmed by immunohistochemical staining of mycobacterial antigens (Fig. 1-O).

Histopathology seen in two lesions after (iii) four weeks of R/S treatment

Emergence of intracellular bacilli both in a nodular and an ulcerative lesion

After four weeks of R/S treatment a strong generalized infiltration and prominent angiogenesis was observed in a nodular lesion (Fig. 2-A). Polymorphonuclear neutrophils (PMNs) and macrophages were the main cellular population of the acute infiltrate throughout the entire lesion (Fig. 2-B). In the upper dermis prominent clusters of eosinophils were revealed (Fig. 2-C). A high power image displayed enlarged macrophages with phagocytosed material (Fig. 2-D). *M. ulcerans* were distributed over the whole lesion sometimes as small extracellular clusters, but mostly internalized by phagocytes (Fig. 2-E; arrows). Moreover, some macrophages engulfed exceptionally large numbers of bacilli until the whole cytoplasm was densely packed, appearing as globi-like structures (Fig. 2-E; arrowhead). Macrophages could be observed while establishing close contact between their cell membrane and single bacteria (Fig. 2-F) and phagocytosing them (Fig. 2-G), respectively. At higher magnification, phagosomes containing densely packed rods could easily be spotted inside these macrophages (Fig. 2-H).

In an early ulcerative lesion, four weeks antibiotic therapy led to the development of immense cellular infiltration surrounding the core necrosis (Fig. 2-I). Adjacent to the necrosis, the infiltrate consisted mainly of PMNs and macrophages (Fig. 2-J) whereas infiltration further away displayed a chronic pattern with lymphocytes and some Langhans' giant cells (Fig. 2-K). Formation of granulomatous structures was identified towards the excision margins (Fig. 2-L) sometimes containing foci of eosinophils (Fig. 2-M). Numerous huge bacterial clusters remained inside the completely destroyed tissue (Fig. 2-N). PMNs were found invading this area in vast numbers and encountering some of the bacterial foci (Fig. 2-O). Bacilli appeared still rod-shaped and the great majority were found to be extracellular (Fig. 2-P), nevertheless phagocytes comprising internalized bacteria could also be detected in distance to the main bacterial burden (Fig. 2-Q).

In the four weeks treated nodule, numerous CD68+ antigen presenting cells, mostly revealing macrophage morphology, carried phagosomes with mycobacterial material (Fig. 3-A). On the other hand mycobacteria were never observed being internalized by Elastase+ PMNs (Fig. 3-B). Comparatively few CD68+ macrophages with phagocytosed mycobacteria were found in the four weeks treated ulcerative lesion

and these cells had already developed karyopyknosis, a sign of apoptosis (Fig. 3-C). As in the nodular lesion, there was no evidence for Elastase+ PMNs containing intracellular bacilli, moreover, PMNs and macrophages were not co-localized in the infiltrate (Fig. 3-D).

Mixed acute cellular infiltration in the nodule

For a detailed characterisation of the cellular immune response inside the nodule treated during four weeks with R/S, IHC was performed on serial sections. Elastase+ PMNs (Fig. 4-A), CD56+ natural killer cells (NK cells; Fig. 4-B) and CD68+ macrophages (Fig. 4-C) were almost equally abundant. Only few CD4+ T lymphocytes (Fig. 4-D) and some more CD8+ T lymphocytes (Fig. 4-E) were observed. Single CD20+ B lymphocytes were distributed within the mixed infiltration (Fig. 4-F). Large amounts of membrane bound and soluble CD14, a pattern recognition receptor expressed by different cell types such as macrophages, were displayed throughout the lesion (Fig. 4-G). CD1a+ Langerhans dendritic cells of the epidermis residing inside the papillary dermis (Fig. 4-H) as well as numerous S100+ dermal dendrocytes distributed within the infiltration (Fig. 4-I) could be detected. Moreover, the infiltrated deeper dermis was interspersed with considerable amounts of Ki67+ proliferating leukocytes and hyperproliferative foci (arrows), respectively (Fig. 4-J). In contrast, in the untreated lesion only destroyed remnants of acute response leukocytes such as PMNs (Fig. 4-K), macrophages (data not shown) and NK cells (data not shown) were observed inside the necrosis. The surrounding tissue stained negative for those cells (data not shown). Mild infiltrates of CD3+ T lymphocytes were found around glands and vessels in vicinity to the necrotic core (Fig. 4-L) mainly appearing to be CD4+ whereas CD8+ staining was entirely negative (data not shown). No CD20+ B lymphocytes were present (data not shown) and only single dermal dendrocytes could be spotted (Fig. 4-M).

Distinct bands of inflammatory leukocytes and early granuloma formation in the ulcer

The early ulcerative lesion showed distinct infiltration zones in vicinity to the necrotic rim of the excised specimens after four weeks R/S treatment, with each zone having its characteristic cellular subsets (Fig. 5a).

The first (that is the inner) belt surrounding the necrotic lesion core displayed acute cellular infiltrates mainly consisting of Elastase+ PMNs (Fig. 5b-A) and was marked off from the second (that is the outer) belt by a line of CD56+ NK cells (Fig. 5b-B) with the majority expressing Ki67, indicating strong proliferative activity (Fig. 5b-C). In the second belt strong positive signals for membrane bound and soluble CD14 were obtained (Fig. 5b-D) and towards the outer margins of this zone increasing numbers of both CD8+ T cells (Fig. 5b-E) and CD4+ T cells (Fig. 5b-F) emerged. CD20+ B lymphocytes were much less frequent in this region, but some small foci were observed in the upper dermis (not shown). Similar to the untreated nodule, high levels of CD1a+ Langerhans dendritic cells were abundant both inside the epidermis and the upper dermis close to vessels (not shown). Additionally, S100+ dermal dendrocytes resided in the outer leukocyte belt around the necrosis (Fig. 5b-G).

For direct comparison, IHC was performed on an untreated ulcerative lesion. In the lesions core and its surroundings staining for PMNs (Fig. 5b-H), NK cells (data not shown) and macrophages (Fig. 5b-I) showed, similar as in the nodule, only cellular debris. Staining for dermal dendrocytes and B lymphocytes was entirely negative (data not shown). Mild infiltrates of CD3+ lymphocytes with apoptotic phenotype, most of them revealing a CD8+ phenotype (Fig. 5b-J), were found around the necrosis. In the periphery, tissue was focally heavily infiltrated with CD8+ T lymphocytes and also some small CD20+ B lymphocyte foci were observed (data not shown).

The dermis of the macroscopically healthy appearing margins of the antibiotic treated lesion contained granulomatous structures primarily built by CD8+ T lymphocytes (Fig. 6-A). CD4+ T lymphocytes were spread inside in smaller numbers (Fig. 6-B) and CD20+ B cell foci could be identified at the rim (Fig. 6-C) of these aggregates. A huge proportion of lymphocytes stained positive for the activation marker CD45RO (Fig. 6-D) and many leukocytes were identified expressing the proliferation marker Ki67 (Fig. 6-G). CD68+ antigen presenting cells were scattered throughout the cellular aggregates (Fig. 6-E), not yet developing into foamy, multinucleated Langhans giant cells (Fig. 6-E_{insert}) as observed after eight weeks R/S treatment [25]. A similar pattern was detected for the distribution of S100+ dermal dendrocytes (Fig. 6-F). Interestingly, dense CD20+ B cell accumulations, often in vicinity to sweat glands, could be identified (Fig. 6-H) and a subset appeared to be Ki67+ due to proliferation (Fig. 6-I).

Discussion

Although provisional WHO guidelines recommend chemotherapy with a combination of R/S for the treatment of all stages of *M. ulcerans* infection, little is yet known about the local and systemic responses to this therapy. In a previous study we observed enormous organized local cellular immune responses as well as destruction of mycobacteria in ulcerative lesions after the recommended eight weeks of antibiotic treatment [25]. This raised more questions regarding the immune processes taking place in the course of R/S therapy and whether there are differences between response patterns in early and late stage disease. On this account, we conducted an in-depth pathological study with samples from a nodular and an early ulcerative lesion excised from patients who had received only four weeks of antibiotic treatment prior to surgery.

It is well established that *M. ulcerans* unlike other mycobacteria appear as extracellular clumps in necrotic connective and adipose tissue [18,27]. Some data also suggest intracellular stages at the periphery of lesions [28]. After four weeks of antibiotic therapy, phagocytosis of large numbers of yet rod-shaped bacilli was observed, possibly still leading to a positive culture [26]. Whilst high counts of mycobacteria were detected inside phagosomes of macrophages in the nodule, most bacilli were still located deep inside the necrosis as extracellular clumps in the ulcer. It appears that the extended necrosis in the ulcerative lesion delays leukocyte entry into the bacterial focus.

In lepromatous leprosy, intracellular bacilli are known to form structures named globi inside histiocytes which are - together with neurotropism - a diagnostic feature of this mycobacteriosis [29]. We could show for the first time that macrophages internalize *M. ulcerans* in large numbers during R/S therapy developing a similar intracellular globus-like packing as seen in leprosy biopsies. Furthermore, after eight weeks antibiotic therapy only bacterial debris instead of rod-shaped bacilli was observed [25]. Macrophages thus seem to be capable of degrading ingested mycobacteria during the course of antibiotic treatment.

It can be assumed that mycolactone production is reduced or abolished early after the onset of R/S treatment due to bacterial growth arrest or death, respectively. Additionally, chemical instability associated with the unsaturated side chain is anticipated, and the core mycolactone itself has no cytopathic activity [30]. We

speculate that mycolactone degrades inside the lesion, leading to declining toxin levels, allowing the establishment of a local immune response and survival of macrophages containing intracellular bacteria.

While there is evidence for the formation of local leukocyte infiltration in untreated chronic lesions, lack of pronounced inflammatory responses is still a histopathological diagnostic criterion in BU [17,18,22,24]. In contrast, after four weeks of antibiotic therapy, pronounced leukocyte infiltration was apparent mostly consisting of cells related to acute inflammatory responses like PMNs and macrophages. In the case of the ulcerative lesion, chronic infiltrates were already present. Here the belt-like organization of acute and chronic infiltrates surrounding the necrotic core is indicative for sequential waves of leukocyte influx.

Granuloma formation and proliferating B lymphocyte clusters are indicators for an adaptive immune response, like it has been reported for chronic pulmonary tuberculosis [31]. After four weeks of R/S we found early granuloma formation and small but highly proliferative B lymphocyte clusters in the ulcerative lesion. In comparison, after eight weeks of antibiotic treatment mature granulomas with Langhans' giant cells and large focal B cell aggregations have typically developed [25].

This is the first work reporting huge amounts of intracellular *M. ulcerans* accompanied by a marked local cellular immune response in patients submitted to R/S treatment. The two cases after four weeks of antibiotic therapy presented here are outstanding in terms of leukocyte infiltration as well as host-pathogen interaction and provide a first insight into the immune response heterogeneity of different antibiotically treated BU stages. Nevertheless patient numbers are too small to draw conclusions universally valid for the various forms of Buruli ulcer. Further analysis of stage-specific differences in local immune responses induced by R/S treatment will help to improve treatment strategies.

Acknowledgements

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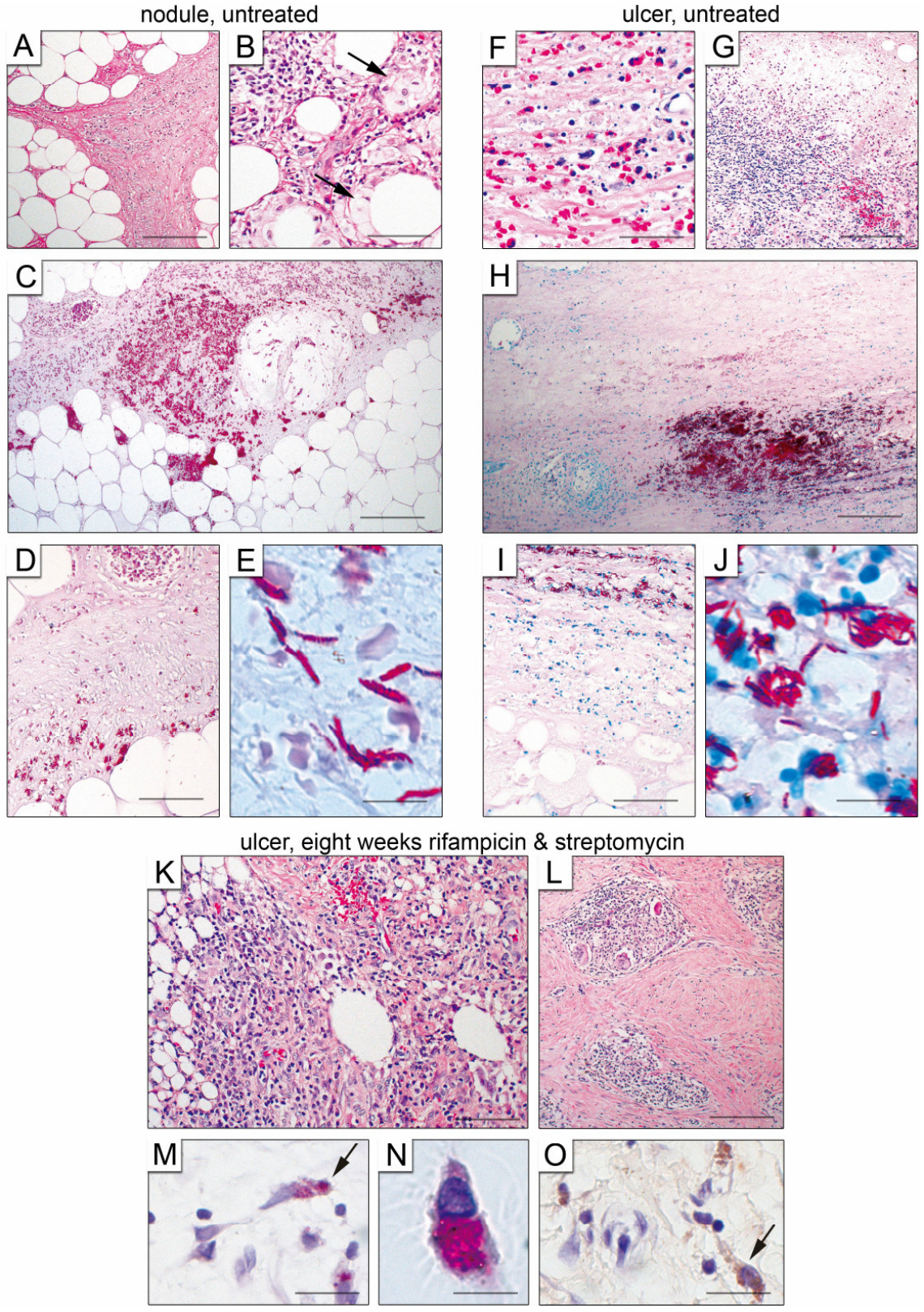
Figures


nodule, untreated
ulcer, untreated
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H
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ulcer, eight weeks rifampicin & streptomycin
K
L
M
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Figure 1: Histopathology of two control lesions without R/S treatment and after eight weeks of chemotherapy, respectively

Histological sections stained with HE (A, B, F, G, K, L), ZN (C, D, E, H, I, J, M, N; counterstain methylenblue) or pAbLep (O; counterstain haematoxylin) of a nodule (A to E) and an early ulcer (F to J) from the same patient without antibiotic therapy and an early ulcer after eight weeks R/S treatment (K to O).

(A) Necrosis characteristic for BU nodules. (B) Slight infiltration and Touton giant cells (arrows) around fat cell ghosts. (C) Focal mycobacterial clusters lying in the centre of a wide-ranging necrosis with great numbers of adipose cell ghosts. (D) Non-detectable cellular infiltration in this section. (E) Extracellular bacteria between erythrocytes from a neighbouring necrotic blood vessel. (F) Developed necrosis with apoptotic cells and broad haemorrhages. (G) Focal acute infiltrates at the necrotic outline. (H) Dense mycobacterial focus within an extensive necrotic area affecting both connective and adipose tissue. (I) Slight acute cellular infiltration in a region containing *M. ulcerans*. (J) PMNs exhibiting apoptotic signs are in close contact to extracellular mycobacteria. (K) Chronic cellular infiltrates in the centre of the lesion. (L) Fully developed granulomas containing Langhans giant cells without central caseous necrosis. (M) Primarily intracellulary located acid-fast bacterial debris can be detected. (N) High power image of a macrophage densely packed with phagosomes containing acid-fast stained material. (O) Immunohistochemical staining with polyclonal antibody pAbLep against mycobacterial surface antigens confirms the ZN staining pattern. Scale bars: 8µm (E, J, N), 20µm (M, O), 40µm (F), 80µm (B, D, I, K), 200µm (A, C, G, H, L).

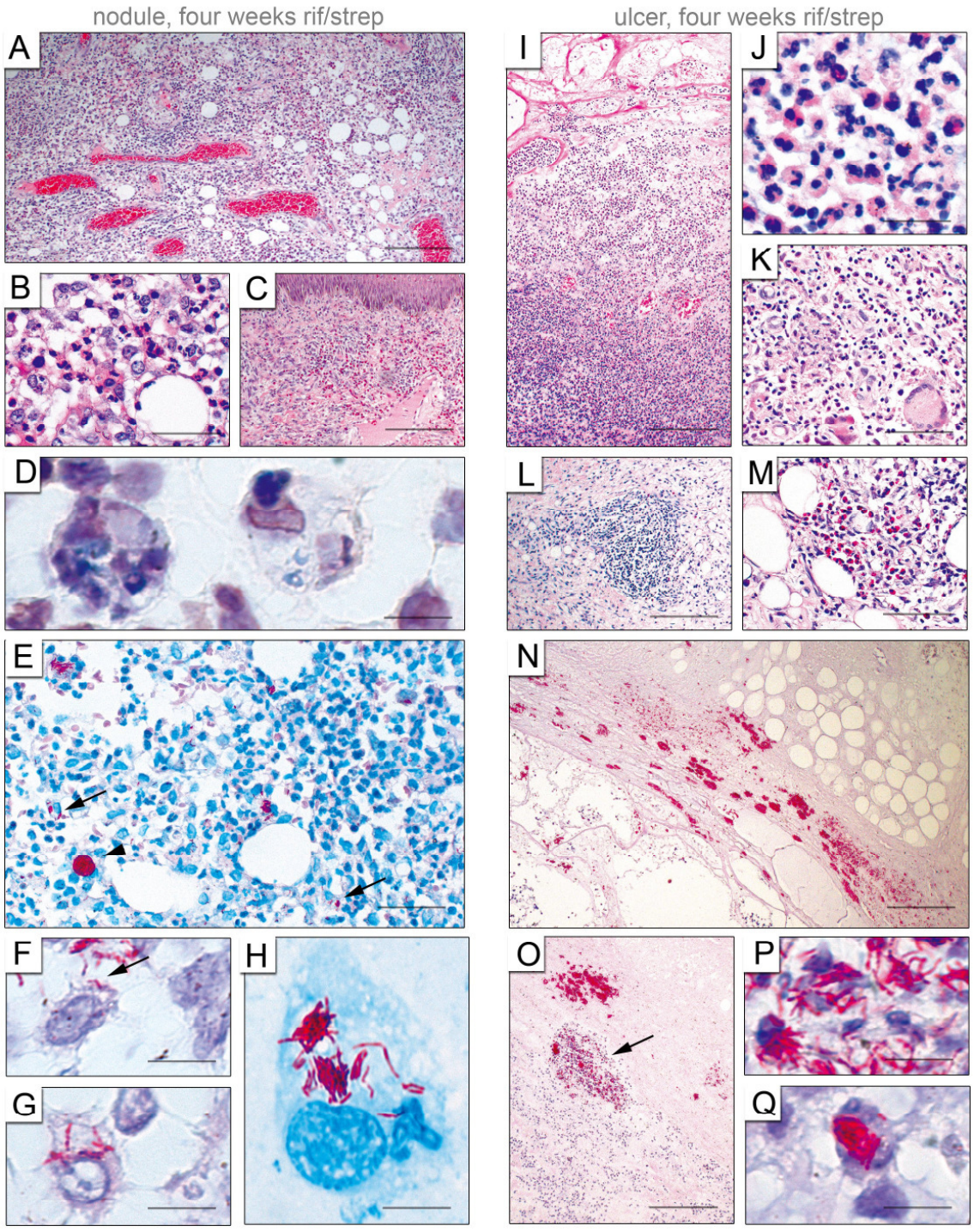
nodule, four weeks rif/strep
ulcer, four weeks rif/strep
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Figure 2: Bacterial load and distribution after four weeks of R/S treatment in a nodular and an early ulcerative lesion

Histological sections stained with HE (A to D and I to M) or ZN (E to H and N to Q; counterstain methylenblue) of a nodule (A to H) and an early ulcer (I to Q) after four weeks R/S treatment.

(A) Extensive mixed cellular infiltration into the necrotic area with neovascularisation and vasodilatation can be found in the whole lesion. (B) PMNs and macrophages are recognized as the prevailing cellular subsets. (C) Focal cluster of eosinophils in connective tissue of the upper dermis. (D) Apoptotic material (here: PMNs) is phagocytosed by macrophages. (E) Bacteria are located both extra- and intracellular (arrows). Infiltrating leukocytes are devoid of apoptotic or necrotic features. (F) Single bacteria become attached to the macrophage membrane (arrow) and (G) subsequently are internalized. (H) High power image of a macrophage with phagosomes containing *M. ulcerans*. (I) Exceeding mixed cellular infiltrates surrounding the expanded necrotic area. (J) Inner belt of infiltration primarily consists of PMNs. (K) Outer belt of chronic infiltration and some Langhans giant cells. (L) Towards the excision margins early stages of granuloma are revealed. (M) Focal cluster of eosinophils together with granuloma formation. (N) In an early ulcerative lesion large cluster of bacteria are still present after four weeks R/S therapy. (O) Substantial cellular infiltrates inside the necrotic area reach the bacterial foci (arrow). (P) PMNs in contact with bacteria reveal apoptotic signs. (Q) Some macrophages can be observed harbouring phagocytosed rod-shaped mycobacteria.

Scale bars: 4µm (H), 6µm (D), 8µm (F, G, P, Q), 20µm (J), 40µm (B, E), 80µm (K, M), 200µm (A, C, I, L, N, O)

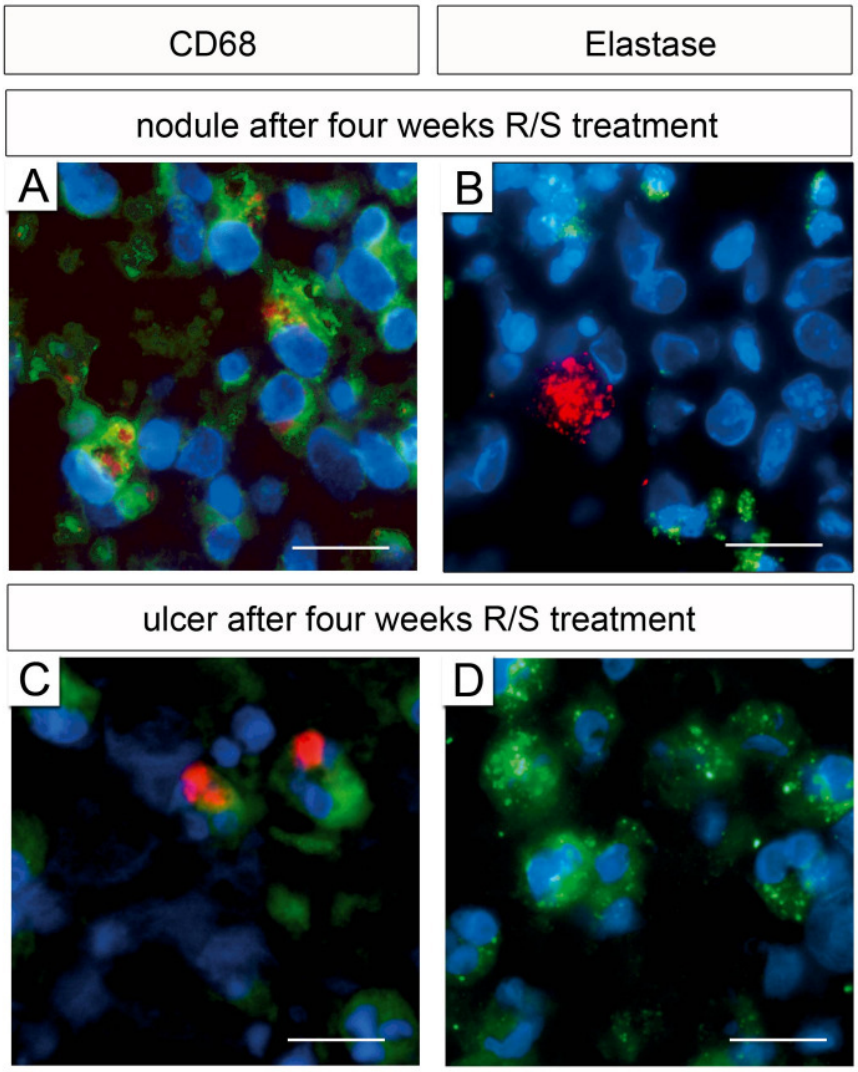
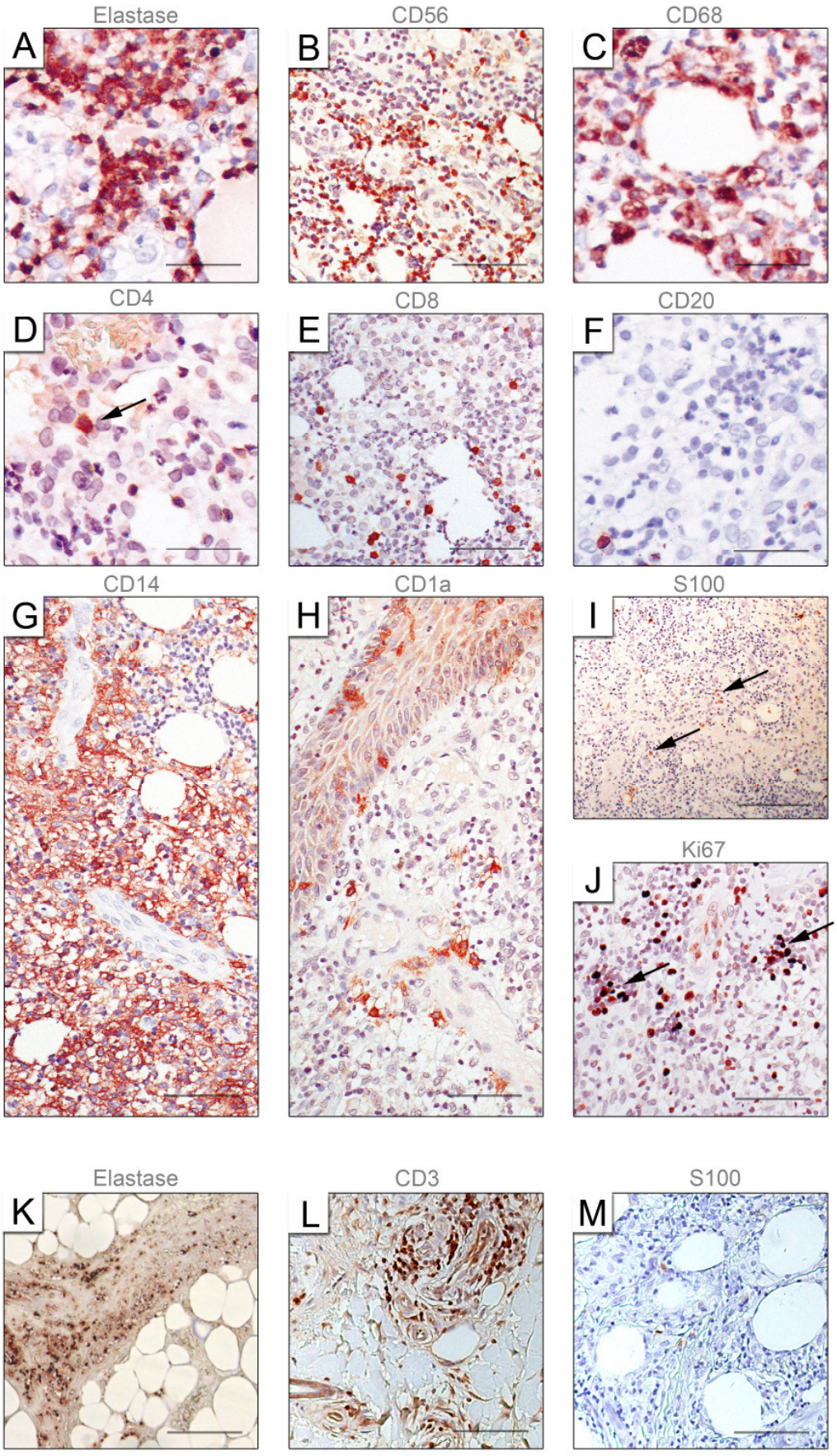


Figure 3: Fluorescence IHC displays rod-shaped mycobacteria residing intracellulary inside macrophages

Immunofluorescent staining for cellular markers (CD68 [A, C] or Elastase [B, D]), polyclonal antibody against mycobacterial antigens (pAbLep) and DAPI in sections from a nodule (A, B) or an ulcer (C, D) treated during four weeks with R/S.

(A) CD68+ antigen presenting cells with macrophage appearance carrying numerous phagosomes containing mycobacteria. (B) PMNs show no co-localization with or internalization of bacterial material. (C) Some slightly apoptotic macrophages comprise intracellular bacteria. (D) No bacteria can be detected inside PMNs. Scale bars: 8µm.


Elastase
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CD56
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CD14
G
CD1a
H
S100
I
Ki67
J
Elastase
K
CD3
L
S100
M

Figure 4: IHC with cellular markers after four weeks R/S treatment and in an untreated nodule

Immunohistochemical staining for cellular markers (red-brown) with haematoxylin as counterstain (blue) in serial sections of a nodule after four weeks R/S therapy (A to J) and an untreated one (K to M), respectively.

(A to C) Acute immune response can be observed consisting of Elastase+ PMNs (A), CD56+ NK cells (B) and CD68+ macrophages (C). (D to F) CD4+, CD8+ and CD20+ lymphocytes are scarce. (G) Macrophages express vast amounts of either surface-bound or soluble CD14. (H) CD1a+ Langerhans dendritic cells migrate from and/or to the epidermal layer. (I) S100+ dermal dendrocytes are distributed over the infiltrate (arrows). (J) A large proportion of the infiltrate are Ki67+ i.e. proliferating cells. Numerous hyperproliferative centres can be observed (arrows).

(K) Only debris of elastase+ PMNs remains inside the necrosis. (L) Focal slight CD3+ T lymphocyte infiltrates is observed around glands and vessels in the subcutaneous tissue. (M) Staining for S100+ dermal dendrocytes is negative apart from very few single cells. Scale bars: 40µm (A, C, D, F), 80µm (B, E, G, H, J, L, M) and 200µm (I, K).

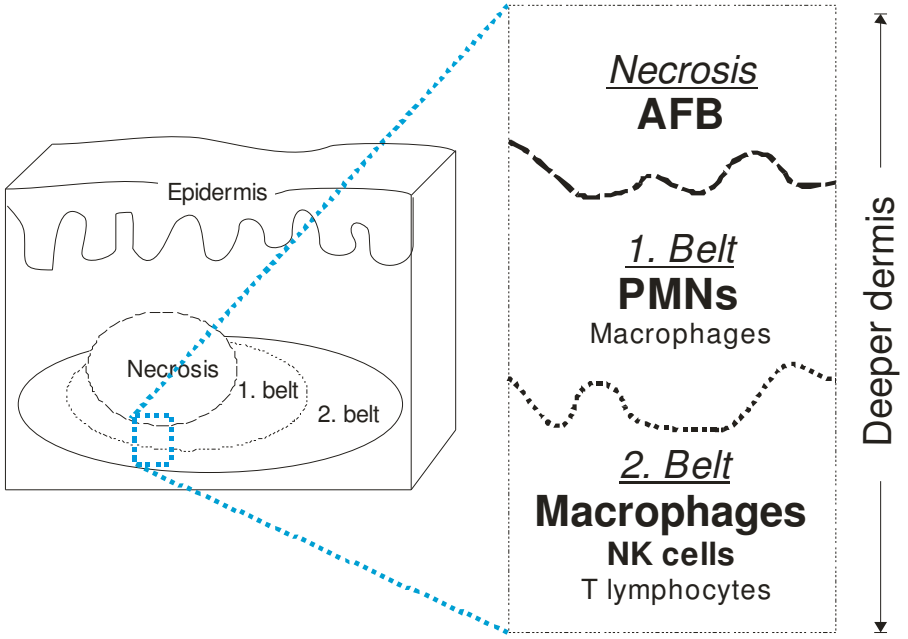

Figure 5a: Schematic illustration of the early ulcerative lesion after four weeks R/S therapy

Infection focus (necrosis), acute (1. belt) and chronic (2. belt) infiltration.

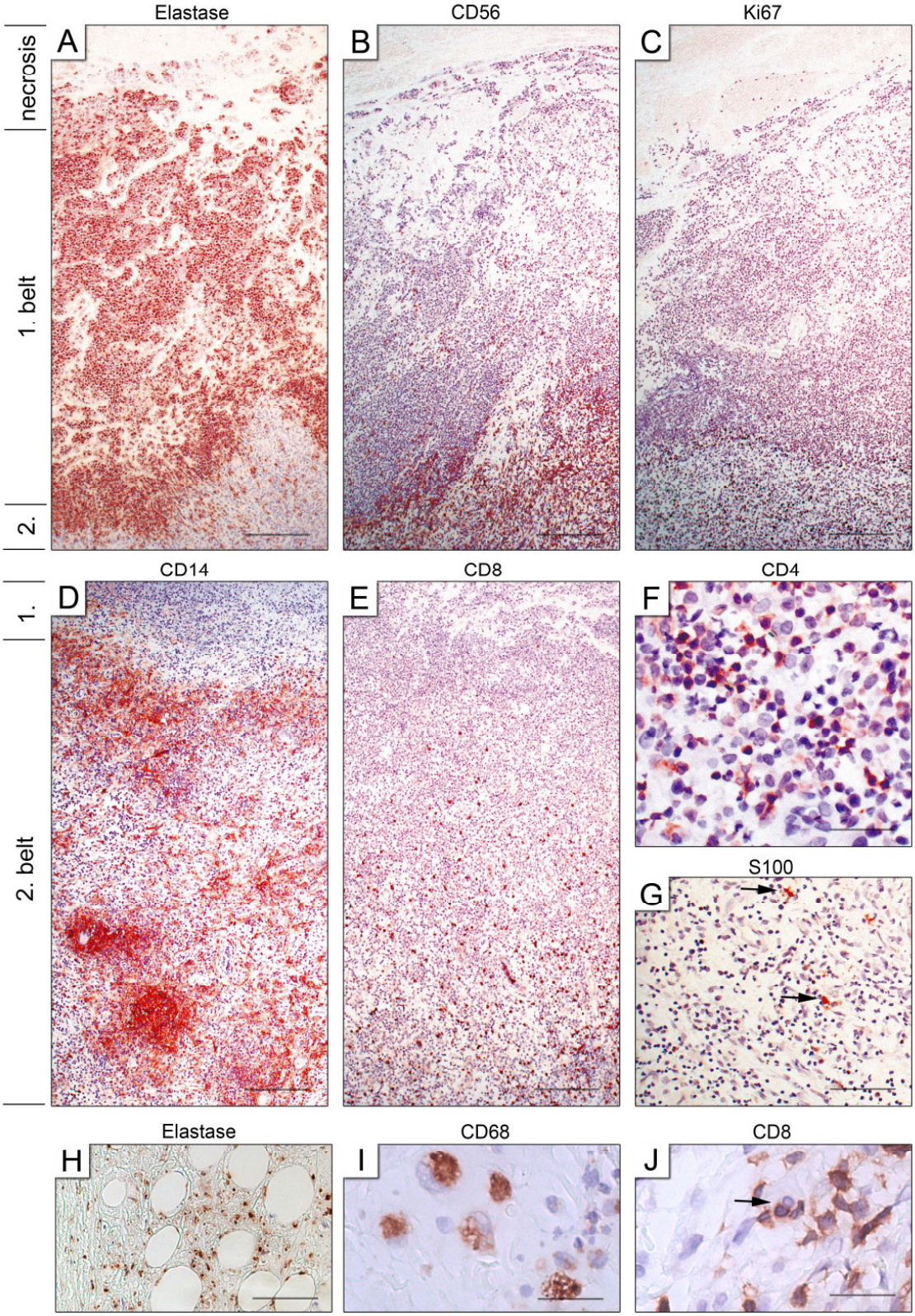
Elastase
CD56
Ki67
necrosis
1. belt
2.
A
B
C
CD14
CD8
CD4
1.
2. belt
D
E
F
S100
G
Elastase
CD68
CD8
H
I
J

Figure 5b: IHC with cellular markers in the core of an early ulcer after four weeks R/S treatment and an untreated control

Immunohistochemical staining with cellular markers (red-brown) and haematoxylin as counterstain (blue) in serial sections from an early ulcer after antibiotic therapy (A to G). (A to C) 1. belt of infiltration and its borders. (D to G) 2. belt of infiltration. (H to J) Control tissue from an untreated ulcerative lesion.

(A) Elastase+ PMNs are the most predominant cell type in the inner layer surrounding the necrosis. (B) CD56+ NK cells build the border between acute and chronic belt and (C) are highly Ki67+ i.e. proliferating. (D) 2. belt of infiltration reveals substantial amounts of membrane bound and soluble CD14. (E) CD8+ and (F) CD4+ lymphocytes are present in this area with rising abundance towards lesion margins. (G) Dispersed S100+ dermal dendrocytes can be observed. (H) Elastase+ staining reveals cellular debris of PMNs. (I) CD68+ macrophages are present in low amounts and show clear signs of apoptosis and necrosis. (J) CD8+ T lymphocytes surrounding the necrotic core undergo apoptosis. Scale bars: 20µm (I, J), 40µm (F), 80µm (G, H, L, M) and 200µm (A, B, C, D, E, K).

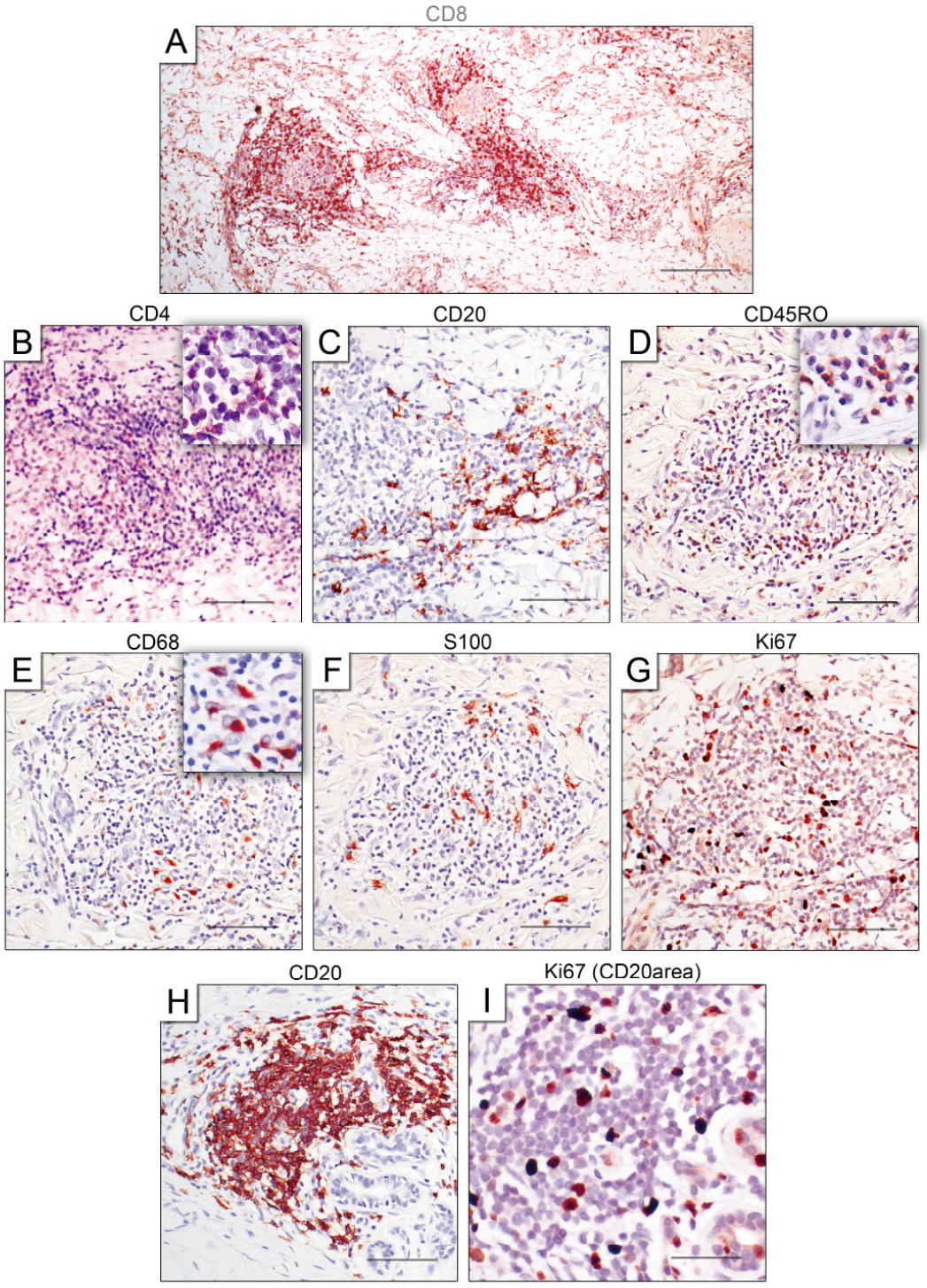
CD8
A
CD4
B
CD20
C
CD45RO
D
CD68
E
S100
F
Ki67
G
CD20
H
Ki67 (CD20area)
I

Figure 6: Early granuloma formation in the healthy appearing margins of an early ulcer after four weeks R/S treatment

Immunohistochemical staining for cellular markers (red-brown) with haematoxylin as counterstain (blue) on serial sections from an early ulcer.

(A) Low power image showing the architecture of granulomas. A large outer layer with CD8+ T lymphocytes being the predominant cell subset surrounds a small non-necrotic core. (B) CD4+ lymphocytes are much less abundant. (C) CD20+ B lymphocytes can be found near the outer rim of granulomas. (D) A great proportion of lymphocytes stain positive for the activation marker CD45RO. (E) CD68+ antigen presenting cells are present without forming a concrete centre not yet starting to become foamy and multinucleated (insert). (F) S100+ dermal dendrocytes and (G) Ki67+ proliferating cells are displayed. (H) In vicinity to vessels and glands dense clusters of CD20+ B lymphocytes are developing. (I) Ki67+ staining reveals proliferative activity inside these lymphocyte foci. Scale bars: 40µm (B_{inset}, D_{inset}, E_{inset}, I), 80µm (B to H), 200µm (A).

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CHAPTER 6

Local immune responses and bacterial killing in early stage Buruli ulcer lesions during treatment with rifampicin and streptomycin

Daniela Schütte[1], Bernard Carbonelle[2], Sebastian Lukas[3], Mark Wansborough-Jones[4], Gerd Pluschke[1]

[1] *Swiss Tropical Institute, Molecular Immunology, Basel, Switzerland*

[2] *Laboratoire de Bactériologie, CHU (University Hospital) Angers, Angers, France*

[3] *St. Thomas's Hospital Campus, UMDS, London, United Kingdom*

[4] *St. George's Hospital Medical School, London, United Kingdom*

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Abstract

Buruli ulcer is a necrotizing skin disease caused by *Mycobacterium ulcerans.* Major necrosis with abundant clusters of extracellularly replicating mycobacteria and only minor infiltration are characteristic histopathological features of the disease. Mycolactone, a macrolide exotoxin of *M. ulcerans* has cytotoxic activities and plays a key role in the development of this pathology. Current treatment strategies include rifampicin-streptomycin chemotherapy with or without additional surgery and/or skin-graft.

Here we have performed a histopathological analysis of Buruli ulcer lesions excised after 0, 2, 4, 8 or 12 weeks of rifampicin-streptomycin treatment. Both localisation and morphology of acid-fast bacilli and the composition and organization of leukocyte infiltrates were monitored. Mycobacteria started to be internalized by phagocytes already after two weeks of treatment and displayed irregular Ziehl Neelsen staining after eight weeks. Acid-fast debris was still found after 12 weeks of chemotherapy in otherwise Ziehl Neelsen negative areas of sections. Cellular infiltrates were already observed after two weeks of chemotherapy and total leukocyte numbers increased during the further course of the treatment. Initially unstructured infiltrates converted with time into lymphocyte aggregates, associated with granuloma and abscessus formation.

Introduction

Mycobacterium ulcerans causes Buruli ulcer (BU), a necrotizing disease of the skin and the subcutaneous tissue often leading to extensive ulceration [1,2]. The bacterium produces an exotoxin with immuno-modulatory and cytotoxic properties named mycolactone [3,4,5,6], which is thought to be responsible for most clinical symptoms related to *M. ulcerans* infection [7,8,9]. Many sub-tropical countries all over the world are affected, but the largest numbers of cases are reported from sub-Saharan Africa [2,10,11,12]. Children develop the disease more often than adults. Whether this is due to more intense exposure or differences in immune status is unclear, since the mode of transmission of BU and possible environmental reservoir(s) of the pathogen are still under investigation [13].
When left untreated, pre-ulcerative forms of BU - nodules, plaques and oedema - break in and form necrotic skin ulcers. Usually the ulcers have deeply undermined edges and enlarge over an extended period of time [12,14].

Until an antibiotic combination therapy with rifampicin and streptomycin (R-S) was recommended by WHO in 2004, surgery was the only available treatment [15,16]. A clinical trial and observational studies have yielded promising results with the combination chemotherapy [17,18]. In a previous study we showed that the treatment of patients with ulcerative BU lesions with R-S leads to the development of highly structured, ectopic lymphoid tissue [19]. Furthermore, our histopathological analyses have provided evidence for a different response pattern in pre-ulcerative lesions (Schütte et al, in press). Therefore we investigated in the present study histopathological changes in early BU lesions – plaque and nodules – after 0, 2, 4, 8 and 12 weeks of R-S treatment and compared results with data from ulcerative lesions. Samples analysed were collected during a clinical trial assessing clinical efficacy of R-S treatment [17]

Material and Methods

Study population

Skin tissue samples used in the present study were collected during a clinical trial performed in 2004 assessing clinical efficacy of rifampicin-streptomycin treatment of early BU lesions [17]. In brief, patients with clinically diagnosed early BU infection (nodules or plaques) were recruited from two endemic districts in Ghana. Only patients older than 15 years with a single lesion of maximum 10 cm in diameter were included after having given written consent. Clinical diagnosis was confirmed retrospectively by culture, histopathology, visible acid fast bacilli (AFB) and/or positive *IS2404* based PCR. Lesions were excised after 0, 2, 4, 8 and 12 weeks of daily administration of R-S, respectively, and tissue specimens equally split for microbiological and histopathological analysis.

Histology and immunohistochemistry

Tissue samples were stored in formalin after surgical excision for various durations until committed to paraffin embedding. Histological and immunohistochemical staining procedures were performed as previously described [19]. In brief, serial sections were stained with haematoxylin-eosin (HE) or monoclonal antibodies specific for the leukocyte markers elastase, CD56, CD20, CD3 and CD68. Furthermore, staining with Ziehl Neelsen (ZN) and polyclonal anti-leprae antibodies (cross-reactive with mycobacterial antigen) was performed to monitor distribution, localization and morphology of *M. ulcerans*. Pictures were taken with a microscope equipped with a camera (Leica Microsystems GmbH) or with MIRAX scan (Carl Zeiss AG). Images were saved using Application Suite (Leica Microsystems GmbH) or MIRAX Viewer and processed with Adobe Photoshop® CS.

Results

Leukocyte infiltration and aggregation during antibiotic treatment

An overview over a representative sample taken after eight weeks of chemotherapy displays histopathological features characteristic for antibiotic treated early BU lesions (Figure 1). Three main areas can be distinguished: sub-epidermal infiltrate, necrosis and deep dermal infiltrates (Fig.1-A). The sub-epidermal layer usually contained polymorphonuclear neutrophilic leukocytes (PMNs) and lymphocyte accumulations, granulomas and granulation tissue (Fig.1-A1). In all samples varying degrees of necrosis were observed (Fig.1-A2). In the subcutaneous region very often cellular infiltration and angiogenesis at exceedingly high levels were present (Fig.1-A3), revealing histological patterns characteristic for healing processes within the severely affected tissue. Secondary tissue damage such as neuritis (Fig.1-B1) or extensive haemorrhages due to destruction of vessel epithelia (Fig.1-B2 and -B3) were observed. On the other hand slight eosinophilia and presence of Langhans' giant cells were markers for transition into a healing process (Fig.1-B4 and -B5). Strong leukocyte infiltrations cumulated in abscessus formations (Fig.1-C1), occasionally forming purulent centres (Fig.1-C2). Abscesses were of elastase+ PMN origin (Fig.1-C3). As an element characteristic for chronic cellular immune responses, Granuloma formation with Langhans' giant cells was found in several lesions of different treatment stages (Fig.1-D1). More commonly dense lymphocyte clusters, sometimes covering large areas of the sub-epidermal tissue, were observed (no 2, 9, 15; Table 2) (Fig.1-D2). These were present in all but three samples and consisted primarily of CD20+ B lymphocytes (Fig.1-D3), but also scattered CD3+ T lymphocytes (not shown).

The three lesions excised from untreated patients contained only low numbers of leukocytes, which were largely (>90%) apoptotic or destroyed. In contrast, the majority of lesions excised after 2 to 12 weeks of R-S treatment showed acute (14/15) and chronic (13/15) infiltrates (Table 1). A high proportion of lesions contained granulomas and abscessus-like PMN accumulations. Moreover, dense lymphocyte congregations were found in every treatment group.

Mycobacterial viability, localisation and morphology during chemotherapy

Bacterial numbers determined by microscopy and culture of tissue homogenate were compared to study loss of viability of *M. ulcerans* during the course of antibiotic therapy (Table 2). While after two weeks of treatment colony forming units (cfu) were still found in all samples analysed, all specimens taken after 4, 8 and 12 weeks of R-S treatment were culture negative. In contrast, bacterial counts in tissue homogenates determined by microscopy were relatively stable over the entire period of R-S treatment (Table 2). In one culture negative sample, AFB were not found in the tissue homogenate, but in the tissue sections. Another sample was positive for AFB in the homogenate, but negative in histology. These discrepancies may be explained by the focal distribution of *M. ulcerans* within the lesions. Overall numbers of AFB found in the tissue sections were not significantly decreasing over time (Table 3). Clumps and/or single extracellular AFB were observed. In addition, in most samples variable numbers of bacteria were located intracellulary within phagocytes. The ratio between extracellular and intracellular bacteria declined with increasing treatment duration (Table 3). Bacterial localisation and morphology at different time-points of antibiotic treatment are shown for representative samples in Figure 2. Already after two weeks, strong leukocyte infiltrations into regions of bacterial burden were detected at the hypoxic rim of the central necrosis (Fig.2-A). PMNs were very abundant in areas of high bacterial burden and had phagocytosed rod-shaped bacteria (Fig.2-A1), whereas AFB-containing macrophages were less frequent at this stage (Fig.2-A2). Some phagocytes carried globi-like mycobacterial aggregations commonly observed in leprosy (Fig.2-A3). At four weeks of R-S therapy solid-stained clumps of AFB were observed both inside the necrosis (Fig.2-B) and the neighbouring infiltrated connective tissue (Fig.2-B1). PMNs (Fig.2-B2) and macrophages (Fig.2-B3) with internalized bacteria were equally abundant. Results from ZN stainings were confirmed by immunohistochemical staining of mycobacterial antigen (Fig.2-C and -C1). In the group of patients treated for eight weeks sections frequently revealed abscessus formation around bacterial clusters and their phagocytosis (Fig.2-D). At this stage extracellular mycobacterial clusters inside the necrosis revealed irregular ZN staining (Fig.2-D1). Decomposed intracellular bacteria were found primarily within macrophages (Fig.2-D2) and only occasionally within PMNs (Fig.2-D3). Immunostaining disclosed the same localisation of extracellular mycobacterial antigen as ZN positive bacterial structures (Fig.2-E), but antigen

staining remained in contrast to ZN staining solid (Fig.2-E2). After twelve weeks of antibiotic treatment, one sample contained no bacterial clumps but only few extracellular bacilli (Fig.2-F and -F1). ZN positive bacterial debris was found in the cytoplasm of macrophages (Fig.2-F2) and PMNs (Fig.2-F3) in the form of mycobacterial debris. Interestingly, immunostaining sometimes uncovered in serial sections antigen clusters that were ZN negative (Fig.2-G). Single bacteria could be stained both in necrotic areas (Fig.2-G1) and inside cellular infiltrations (Fig.2-G2).

Discussion

There may be a constant influx of neutrophils, monocytes/macrophages and lymphocytes to active BU lesions (Oliveira *et al.,* 2005), but high concentrations of mycolactone around the extracellular foci of *M. ulcerans* are driving these cells into apoptosis. In this study we show that antibiotic therapy of BU nodules and plaques leads to local cellular immune responses and phagocytosis of the bacteria. Intracellular bacteria were found already after two weeks of treatment, when culture was still positive. Antibiotic treatment may act rapidly on the bacterial metabolism and interfere with mycolactone production, while complete killing of bacteria takes more time. Similar findings have been reported for multidrug therapy in leprosy [20]. Declining mycolactone concentrations allow infiltrating leucocytes to reach the bacterial foci, where they can enhance the anti-mycobacterial activity of the antibiotics. The fact that cultures turned negative after four weeks of chemotherapy evidences, that the antibiotics are able to sufficiently penetrate necrotic regions to kill the mycobacteria residing there. This observation is emphasized by fading ZN staining at coeval solid immunostaining of bacterial clusters after eight and twelve weeks of therapy. In *M. leprae* infections the so-called morphological index calculated by counting the numbers of solid-staining acid-fast rods, is used as a viability estimation [20]. Development of a similar viability index for *M. ulcerans* based on the bacteria's morphological changes during therapy are currently under investigation.

Extended necrotic areas, typically containing huge extracellular mycobacterial clusters, were still found in the BU lesions even 12 weeks after start of the R-S treatment. While these areas are at least partially washed out after ulceration, tissue debris and huge amounts of mycobacterial antigens and immunostimulators remain in the affected tissue after antibiotic therapy of pre-ulcerative lesions. In this respect chemotherapy differs profoundly from surgical treatment, where necrotic tissue containing the vast burden of mycobacteria is excised. This is a feature most likely triggering the strong leukocyte infiltration and abscessus formation observed in the BU lesions in the course of chemotherapy. Slow resolution of necrotic areas and massive encounter of immune cells with remaining bacterial material may explain why re-activation of plaques and/or oedematous forms of BU is observed in some

patients several weeks after completion of antibiotic therapy. This reaction is often associated with the sensation of heat and pain and a secondary ulceration, but not with the development of new mycobacterial foci (unpublished results) [18].

The massive leukocyte infiltrates found in all lesions analysed here may cause a retardation of the healing process. In knock-out mice which lack macrophages, neutrophils and mast cells, more rapid repair processes and reduced scarring can be observed [22]. On the other hand, macrophages and their cytokines are needed to govern granulation tissue formation which relies on angio- and vasculogenesis [23]. Granulation tissue was observed in most of the samples as were macrophages. Moreover, eosinophils were found in varying numbers in all lesions. These act as regulators of tissue immune microenvironment and modulate the responses from recruited and resident leukocytes [24] .Comparison of our findings with pre-ulcerative early stages of BU presented here with results obtained with ulcerative lesions during R-S treatment revealed commonalities, but also clear differences. After eight weeks of R-S treatment, bacterial debris was barely found in ulcerative lesions and necrotic areas were smaller. In contrast to pre-ulcerative lesions infiltrates contained no neutrophils [19], indicating that the lower burden of mycobacterial antigen and necrotic material permits a faster transition from acute to chronic immune response. Taken together our data indicate that persisting mycobacterial antigens and immunostimulators may cause excessive immuno-stimulation and some healing retardation after successful chemotherapy. Apparent reactivation of the disease in a small proportion of patients after chemotherapy should be carefully analysed, but appears to be related to immunological factors rather than incomplete killing of the pathogen.

Figures

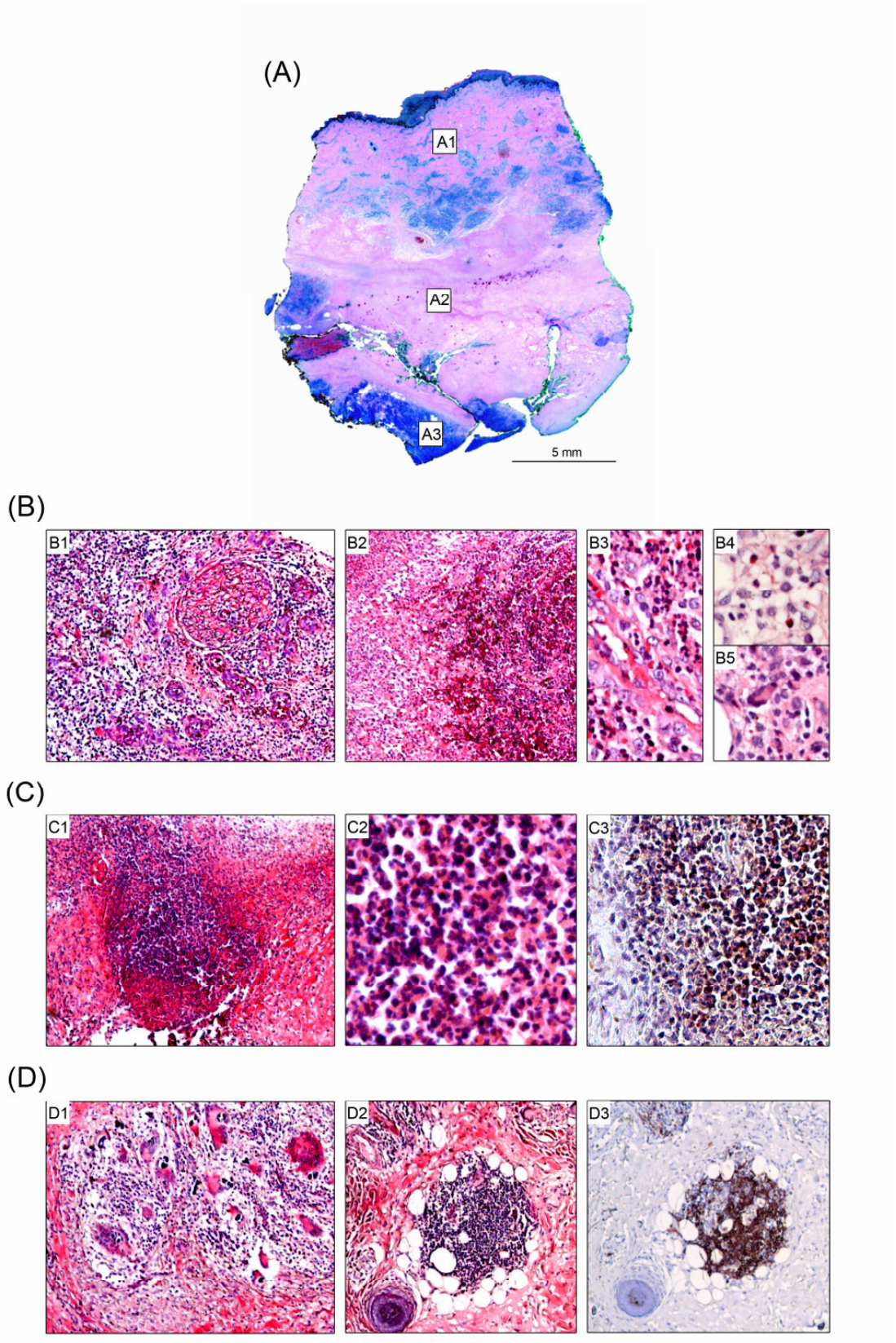


Figure 1. Local histopathological features of early BU lesions in the course of R-S therapy

Histological sections were stained with HE (A, B, C1, C2, D1, D2) and antibodies against elastase (C3) or CD20 (D3).

(A) Overview a representative sample from an early BU lesion after eight weeks of R-S treatment. (B) Bystander tissue damage such as severe neuritis (B1), extensive haemorrhages (B2) and degradation of vessel epithelia (B3). Presence of eosinophils (B4) and Langhans' giant cells (B5). (C1-3) PMNs gathering as abscessus formation with purulent centres. (D) Cellular aggregations in the form of granulomas (D1) and lymphocyte clusters (D2) of B cell origin (D3).

duration of treatment (weeks)	n	Necrosis		total infiltration		leukocyte infiltration				
		▼	▲	▼	▲	acute	chronic	granuloma	abscess	b/t cell aggregates
2	4	0	4	3	1	4/4	4/4	2/4	2/4	3/4
4	3	2	1	2	1	3/3	3/3	3/3	3/3	3/3
8	5	3	2	2	3	5/5	4/5	4/5	4/5	4/5
12	3	2	1	1	2	2/3	2/3	2/3	2/3	2/3

▼necrosis/infiltrate covers ≤15% of the sample surface;
▲necrosis/infiltrate covers >15% of the sample surface

Table 1. Necrotic tissue coverage and leukocyte infiltration pattern during chemotherapy

duration of treatment (weeks)	patient no	CFU[1] culture	BC[2] microscopy	AFB[3] histology
0	1	$1x10^4$	$1x10^9$	+
	2	$1x10^2$	$9x10^7$	+
	3	$1x10^2$	$6.4x10^7$	+
2	4	$4x10^3$	$8x10^7$	+
	5	$1x10^2$	$1.2x10^7$	+
	6	$1x10^4$	$6.6x10^7$	+
	7	$1x10^3$	$1.9x10^8$	+
4	8	0	$2.7x10^8$	+
	9	0	$2.2x10^8$	+
	10	0	$4.9x10^7$	+
8	11	0	$4.8x10^7$	+
	12	0	$2x10^7$	+
	13	0	0	+
	14	0	$1.2x10^8$	+
	15	0	$1.8x10^8$	+
12	16	0	$1.2x10^8$	+
	17	0	$1.2x10^6$	-
	18	0	$1x10^7$	+

[1]colony forming units (per gram of tissue homogenate)
[2]bacterial counts (bacilli per gram of tissue homogenate)
[3]acid fast bacilli (detected in histological tissue sections)

Table 2. Bacterial load and viability in response to antibiotics

duration of treatment (weeks)	Bacterial load[1]		Proportion of extracellular bacteria[2]		
	high	low	> 90%	10-90%	< 10%
0	1	2	3	0	0
2	4	0	2	2	0
4	1	2	1	1	1
8	1	4	1	2	1
12	1	1	1	0	1

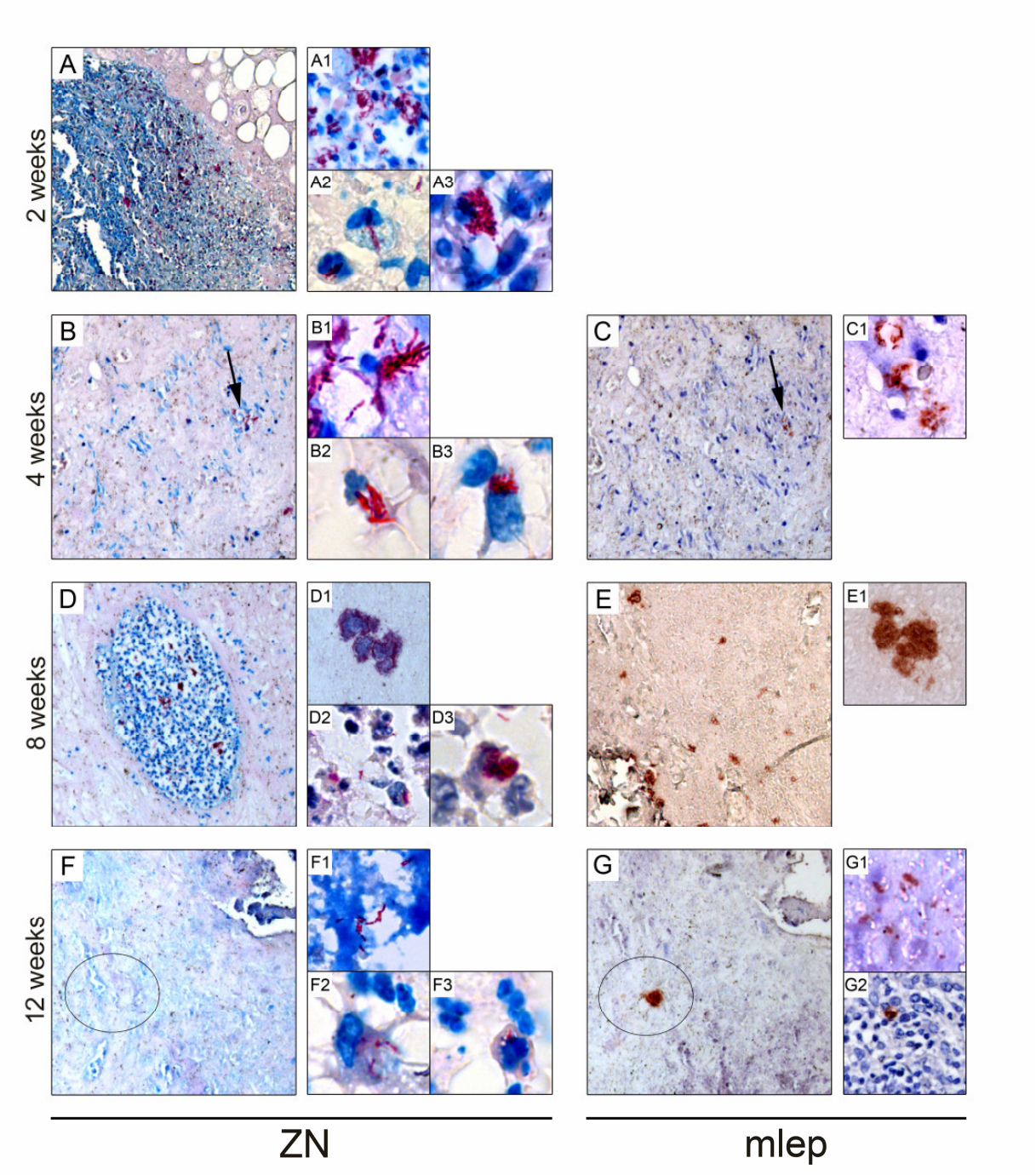
[1]high: large clusters visible at 200x or less
[1]low: small clumps or single bacteria only visible at 600x and more
[2]ratio extracellular/intracellular

Table 3. Histological analysis of number and distribution of *M. ulcerans* during R-S treatment

Figure 2. Localisation and morphological changes of *M. ulcerans* in early lesions after 2, 4, 8 and 12 weeks of chemotherapy

Histological sections were stained with ZN (A, B, D, F) and polyclonal antimycobacterial antibodies against surface antigens (C, E, G).

(A) Strong infiltration into regions of bacterial burden after 2 weeks of R-S therapy. *M. ulcerans* were phagocytosed primarily by PMNs (A1) and to a much lesser extent by macrophages (A2), sometimes forming globi-like structures (A3). (B/C) Bacterial clumps after 4 weeks of R-S treatment. Solid staining AFB were present both extracellularly (B1) and inside PMNs (B2) and macrophages (B3). ZN staining was confirmed by IHC (C1). (D/E) Bacterial morphology after 8 weeks of R-S therapy. Abscessus formation around M. ulcerans (D). Bacterial cluster inside the necrosis revealed irregular ZN staining (D1). ZN positive debris was observed equally inside PMNs (D2) and macrophages (D3). (E) Confirmation of AFB picture through IHC reveals solid antigen staining (E1). (F/G) Appearance of *M. ulcerans* after 12 weeks of R-S administration. Only single extracellular AFB were found inside the necrosis (F1). Both PMNs and macrophages contained mycobacterial debris (F1 and F2, respectively). Immunostaining uncovered ZN negative mycobacterial antigen (G). Single solid staining bacteria were found both in necrotic (G1) and infiltrated (G2) tissue.


A
A1
A2
A3
2 weeks
B
B1
B2
B3
4 weeks
C
C1
D
D1
D2
D3
8 weeks
E
E1
F
F1
F2
F3
12 weeks
G
G1
G2
ZN
mlep

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CHAPTER 7

Phase change material for thermotherapy of Buruli ulcer

Junghanss T[1], Um Boock A[2], Vogel M[1], Schuette D[4], Weinlaeder H[3,] Pluschke G[4]

[1]Section Clinical Tropical Medicine, University Hospital, Heidelberg, Germany

[2]ALES, Bureau Régional pour l'Afrique, Yaoundé, Cameroon

[3]Bavarian Center for Applied Energy Research, Thermal Insulation and Heat Transfer, Wuerzburg, Germany

[4]Dept. Medical Parasitology / Infection Biology, Swiss Tropical Institute, Basle, Switzerland

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Abstract

Background: Buruli ulcer (BU) is an infection of the subcutaneous tissue leading to chronic necrotizing skin ulcers. The causative pathogen, *Mycobacterium ulcerans*, grows best at 30 °C – 33 °C and not above 37 °C. We explored the safety, tolerability and efficacy of phase change material (PCM), a novel heat application system for thermotherapy of BU.

Methodology/Principal Findings: In a prospective observational single centre proof-of-principle trial in Ayos / Cameroon, six laboratory reconfirmed patients with ulcerative Buruli lesions received 28–31 (ulcers ≤ 2 cm) or 50–55 (ulcers > 2 cm) days of thermotherapy with the PCM sodium acetate trihydrate as heat application system. This PCM is widely used in commercial pocket heat pads, it is easy to apply, rechargeable in hot water, non-toxic and non-hazardous to the environment. All patients enrolled in the trial completed treatment. Being completely mobile during the well-tolerated heat application, acceptability of the PCM bandages was very high. In patients with smaller ulcers, wounds healed completely without further intervention. Patients with large defects had skin grafting after successful heat treatment. Heat treatment was not associated with marked increases in local inflammation or the development of ectopic lymphoid tissue. One and a half years after completion of treatment, all patients are relapse-free.

Conclusions/Significance: Our reusable PCM-based heat application device appears perfectly suited to treat BU in endemic countries with limited resources and infrastructure.

Trial Registration. Controlled-Trials.com ISRCTN88392614 [http://www.controlled-trials.com/ISRCTN88392614]

Author Summary

Buruli ulcer is an infection of the subcutaneous tissue leading to chronic necrotizing skin ulcers. The causative pathogen, *Mycobacterium ulcerans*, grows best at 30 °C – 33 °C and not above 37 °C, and this property makes the application of heat a treatment option. We achieved a breakthrough in heat treatment of Buruli ulcer by employing the phase change material sodium acetate trihydrate as a heat application system for thermotherapy, which is widely used in commercial pocket heat pads. It is easy to apply, rechargeable in hot water, non-toxic and non-hazardous to the environment. Six laboratory reconfirmed patients with ulcerative Buruli lesions were included in the proof-of-principle study and treated for four to six weeks. In patients with small ulcers, wounds healed completely without further intervention. Patients with large defects had skin grafting after successful heat treatment. Heat treatment was not associated with marked increases in local inflammation or the development of ectopic lymphoid tissue. One and a half years after completion of treatment, all patients are relapse-free. The reusable phase change material–based heat application device appears perfectly suited for use in remote Buruli ulcer–endemic areas of countries with limited resources and infrastructure.

Introduction

Buruli ulcer (BU) is a chronic necrotizing disease of skin and soft tissue caused by *Mycobacterium ulcerans* [1]. The disease starts as a subcutaneous nodule, papule or plaque that eventually ulcerates and progresses over months to years. In BU lesions, clumps of extra-cellular acid-fast organisms surrounded by areas of necrosis are found primarily in subcutaneous fat tissue [2]. *M. ulcerans* produces a macrolide toxin, mycolactone, which is associated with tissue destruction and local immunosuppression [3]. BU has been reported in >30 countries, but the major burden lies on children living in remote areas of West Africa associated with swamps and stagnant water bodies. Traditionally wide excision of the infected tissue alone was the standard treatment for BU. This is hampered by traumatic interventions, high cost and very high recurrence rates [4]. Chemotherapy with streptomycin and rifampicin is currently re-evaluated as an adjunct treatment to surgery and as a therapy in its own right [5,6,7,8].

M. ulcerans differs from most other pathogenic mycobacteria in that it grows best at 30 – 33 °C and not above 37 °C [9]. This characteristic feature of the pathogen was first used for therapeutic purposes in the early 1970s. Meyers et al. treated 8 patients from Zaire maintaining a temperature of approximately 40 ºC in the ulcerated area for a mean duration of 68 days [10]. There was no evidence of local recurrence during follow-up periods of up to 22 months. Based on this impressive success rate, WHO guidelines listed the application of heat as a treatment option for BU [11]. However, the heat application devices employed so far were impractical in most endemic countries. Here we describe the use of a cheap and easy to apply phase change material (PCM) device suitable for thermotherapy of BU in countries with limited resources.

Methods

Study participants

Eligibility criteria for participants and case definition. All patients between 6 and 30 years of age with an ulcer at the lower or upper arm or leg with a diameter of up to 12 cm suggestive for BU on clinical grounds in the catchment area of the Buruli treatment center Ayos / Cameroon were candidates for inclusion in the study. They were not admitted to the study if any of the following criteria were present: (1) clinical signs and symptoms of communicable diseases other than BU (fever, weight loss, night sweats, persistent cough, jaundice, pulmonary or myocardial dysfunction, CNS involvement, ascites, pleural effusion), (2) clinical signs and symptoms of non-communicable diseases (myocardial, pulmonary, renal, CNS) and (3) inability to confirm BU using laboratory methods.
A BU case was defined as a patient with an ulcer diagnosed as BU on clinical grounds and positive results in at least two of the three laboratory tests (PCR, detection of AFB on microscopy and histopathology) performed.

Laboratory confirmation of clinical diagnosis. On day 0 four swabs from the undermined edges and one diagnostic biopsy were taken from all patients enrolled into the trial on clinical grounds. A second set of biopsies was taken in week 4 of thermotherapy to assess histopathological changes in response to heat treatment. All samples were investigated by microscopy for acid-fast bacilli (AFB) after Ziehl Neelsen (ZN) staining and by IS2404 real-time PCR [12]. Histopathological changes typical for BU [12] were recorded in the initial biopsies and the follow-up biopsies in week 4 of thermotherapy.

Immediately after performing the punch biopsies, tissue samples were fixed in 4% neutral-buffered PFA (paraformaldehyde) for 24h and subsequently transferred to 70% ethanol for short term storage and transport. Biopsies were dehydrated, embedded in paraffin, cut into 5µm thin sections and retrieved on glass slides. After dewaxing and rehydration, sections were stained with haematoxylin/eosin (HE) and ZN. Immunohistochemistry (IHC) was performed with antibodies against Elastase (polymorphonuclear neutrophils [PMNs]; Dako) and CD3 (T lymphocytes; Dako). Staining was performed using Vector NovaRED and haematoxylin.

The setting and location where the data were collected. Volunteers were recruited in the catchment area of the Buruli treatment center at the hospital Ayos / Cameroon, identified by active and passive case detection. The treatment center has a longstanding collaboration with and is supported by Leprosy Relief Emmaus-Switzerland (ALES). It maintains a very well equipped and functioning operation theatre, wards for pre- and postsurgical care, physiotherapy and a school which is of importance because the majority of patients with this disease are children and convalescence after excision of ulcers and skin grafting takes many months in the majority of patients. Dr. A. Um Boock, the director of the ALES Bureau Régional pour l'Afrique, and his team are very experienced in the diagnosis and management of patients with Buruli ulcer, including surgery and skin grafting.

Ethical approval and informed consent. The protocol was approved by the National Ethics Committee of Cameroon and the Ethics Committee of the University Hospital, Heidelberg, Germany. Patients were enrolled in the study only after informed written consent was obtained from them or their care providers.

Interventions

Heat application. Commercially available plastic bags filled with the PCM sodium acetate trihydrate were used. Starters were placed in the bags to initiate the crystallisation process (Fig. 1). Size of filled bags is 21 cm x 15 cm x 2 cm with an average weight of 800 g. The melting temperature of the PCM sodium acetate trihydrate is 58°C. The unique feature of PCM is its thermal energy storing capacity combined with an almost constant temperature during the liquid-solid phase transition. This property is widely used in commercial pocket heat pads.

After cleaning and sterile dressing of the ulcers a heat sensor connected to a data logger (testo 177-T3, testo AG, Lenzkirch, Germany) was placed on healthy skin at the edge of the ulcer. The area of contact between skin and PCM packs was protected by tube gauze and a layer of elastic bandage to lower the PCM working temperature from 58°C to the therapeutic target temperature of 40°C at skin surface (Fig. 1). Temperatures of ≤58°C do not cause burns when not applied for prolonged periods of time. Skin temperatures of up to 43°C were accepted and well tolerated for short intervals of time immediately after mounting the PCM bandage. The affected

skin (ulcer /oedema / induration) plus a safety margin of several centimeters was covered by one to four PCM packs per session depending on the size of the total area to be treated (Fig. 2). The PCM packs were fixed with several layers of elastic bandage. A thermal insulation layer, commercially available to insulate hot water pipes, was used to reduce heat loss to the environment and to reinforce positioning of the PCM packs (Fig. 1). This allowed patients to move around freely. The 24 hours protocol was as follows: 8.00: Clinical progress assessment, cleaning and dressing of ulcers and renewal of PCM-packs, photo documentation at, on average, 3 day intervals. 12.00: Removal of PCM-packs, dressing of the wound to protect from contamination during a 5 hour pause of heat treatment, skin care with fatty cream. 17.00: Additional wound cleaning and dressing, if needed, renewal of PCM-packs. 22.00: Renewal of PCM-packs.

Clinical observations (appearance of the ulcer and the surrounding heat exposed skin, overall clinical assessment of the patient) were recorded daily at the above mentioned time points on case record forms (CRF). Temperature at the skin surface was automatically recorded at 10 minute intervals and stored in a small data logger carried by the patients. Temperature data were transferred daily to a notebook and checked for therapeutic and safety margins (testo software ComSoft 3.4, testo AG, Lenzkirch, Germany).

Patients with small ulcers and without significant oedema (patients 1, 2, 3) received heat treatment for 28-31 days, patients with large ulcers and / or significant oedema (patients 4, 5, 6) for 50-55 days.

Study objectives

In the current study we tested the hypotheses that

(1) PCM-based heat application is safe and comfortable for patients

(2) with PCM based heat application the results of the thermotherapy study of Meyers et al [10] can be reproduced, i.e. primary healing of Buruli ulcer without relapse can be achieved

Primary outcomes

(1) Proportion of patients completing 28-31 days of heat treatment in patients with small ulcers (≤ 2 cm) or 50-55 days in patients with large ulcers (> 2 cm) and ulcers with prominent surrounding oedema
(2) Proportion of patients cured 6 months after completing heat treatment (including skin grafting where necessary). Cure is defined as complete closure of the wound by epithelialisation or scarification or by skin graft.
(3) Proportion of patients who are recurrence free 18 months after completing heat treatment

Secondary endpoint

Histopathological responses in week 4 of thermotherapy compared to reference samples at day 0.

Results

Participant flow

Seven patients with ulcers suggestive for BU on clinical grounds were recruited by active and passive case detection. In six of the seven patients enrolled the diagnosis was laboratory confirmed.

Protocol deviations

We extended the total duration of heat application of large ulcers (> 2 cm) and ulcers with prominent surrounding oedema from 4 week to 50-55 days and did not, as originally planned, treat small and large ulcers equally for 4 weeks only. This was done even though all ulcers appeared clinically healed after 4 weeks of heat treatment, independent of size and surrounding oedema. This decision was taken on the basis of the results of the punch biopsies in week 4 of thermotherapy showing residual AFB with intact rod-shaped appearance.

Recruitment and follow-up

Eligible patients were recruited between February 28, 2007 and March 3, 2007. Patients stayed in the hospital during the course of heat treatment and thereafter until the wound was closed (patients with small ulcers; patients 1, 2, 3, 4,) or skin grafted (patients with large ulcers; patient 5 and 6). All patients were followed up until 18 months after completion of heat treatment.

Baseline data

The age range of the seven patients enrolled was six to 21 years. Three patients had single ulcers on the upper and four had single ulcers on the lower extremities. Medical history and physical examination revealed no significant health problem other than BU. In six out of seven patients enrolled in the study on clinical grounds, diagnosis was laboratory confirmed. The unconfirmed patient was excluded from the analysis (Fig. 2).

Outcomes

All patients enrolled into the trial completed treatment. In all patients temperatures at the lesion and over a wide margin of healthy looking skin were maintained above ≥

39°C for between 8.4 and 13.2 hours and ≥ 40°C for between 4.4 and 9.3 hours per day (Fig. 2). Undermined margins collapsed between day 1 and day 3. Epithelialization started in all patients between 4 and 11 days after the start and was almost completed in patients 1, 2, and 3 at the end of heat treatment (Fig. 2 and Fig. 3). In particular in patients with oedematous lesions (patients 4, 5) white discharge from ulcers was observed during initial treatment for various lengths of time. The two patients with large defects (patients 5 and 6) had skin grafting after completion of heat treatment (Fig. 3B).
All six reconfirmed patients were healed and relapse-free 18 months after completion of treatment.

In the punch biopsies taken prior to start of treatment, histopathological changes characteristic for BU, such as fat cell ghosts, deep dermal necrosis and/or psoriasiform epidermal hyperplasia, were found in six patients (Fig. 2). All patients yielded positive semi-quantitative IS2404 real-time PCR results. AFBs were detected in swabs or punch biopsies of 4 out of 6 patients included in the study.

Analysis of serial sections of punch biopsies taken at day 0 and in week 4 of thermotherapy showed, that heat treatment was not associated with marked increases in local inflammation, the development of ectopic lymphoid tissue or haemorrhages. At both time points small numbers of both polymorphonuclear cells as members of the innate and T cells as members of the adaptive immune system were present, with polymorphonuclear cells mainly located around necrotic areas and T cells more confined to areas close to vessels in the upper dermis. Only the lesion of patient 3 contained both on day 0 and in week 4 of thermotherapy mixed cellular infiltrates, which were much more pronounced than in typical untreated BU lesions.

Safety and tolerability of PCM-based heat treatment, adverse events
The heat treatment procedure was very well tolerated by all patients. Patients with one (patients 1, 2, 3, 4) and with two PCM packs (patient 5) could move around freely and did not feel unacceptably disturbed during their daily activities nor during sleep at night. Patient 6 with four PCM packs also walked with acceptable restrictions and slept largely undisturbed. None of the patients and their guardians requested termination of treatment at any time. Temperatures between 40 - 43°C were

observed only for short intervals of time immediately after mounting of the PCM packs without causing unacceptable discomfort. Only initially a few small blisters were occasionally observed. With a simple patient-controlled method the therapeutic target temperature of 40°C at skin surface was quickly reached and maintained without further side effects.

Discussion

Successful treatment of BU with heat has been reported in individual patients and small case series since 1950 [10,13,14,15]. This has not been carried further into clinical research and practice due to the fact that available heat application systems were cumbersome and not suited for use in developing countries. We achieved a break through by employing PCM packs as a cheap heat application system which is rechargeable in hot water, non-toxic and non-hazardous to the environment. In this proof-of-principle study we demonstrated that our heat application system is easy to use and allows the patient to move freely.

Family members and the hospital community accepted the treatment very well and favoured it over other treatments currently offered (surgery, antibiotics). Nurses quickly adopted the techniques of mounting the PCM packs and of recharging the packs in boiling water. The only side effects observed were sensation of excessive heat for a short period after applying the PCM packs. Lowering of the temperature at the skin surface by an elastic bandage interposed between tube gauze and PCM packs reliably prevented skin irritation and development of blisters, which may occur if the initial temperature at skin surface is less rigorously controlled.

With our PCM-based heat application system we reproduced the excellent results of the thermotherapy study of Meyers' group in 1974 [10] with significantly shorter heat application times both with respect to length of heat treatment per day (close to 24 hours [39°C – 40.5°C] vs a mean of 10 hours, range 8.4 – 13.2 hours [≥39°C]) and to total heat application time (28 to 115 days vs 28 to 55 days). Since both systems worked at the same temperature range measured at skin surface, the minimum length of heat application to achieve healing of BU appears to be in the range of our heat treatment schedule or even shorter.

The initial clinical improvement of ulcerative lesions in our series was as fast as in the patient series of Meyers et al. As early as three days after initiation of heat treatment undermined ulcer margins collapsed and the skin attached to the underlying subcutaneous tissue with re-epithelialization starting at the edges. Discharge of the wound decreased over various lengths of time. Firm attachment of the affected skin

was complete only after discharge stopped. By using heat treatment alone no viable tissue is lost and even the overarching margins at undercutting edges are often rescued. Lesions were clinically inactive in all of our patients with very good granulation and re-epitheliazation responses after 28 days of heat treatment. In one of our patients (patient 6) non-viable tissue extended far beyond the ulcerated area, which had to be excised before skin grafting. In this patient and one other patient with a large defect (patient 5) skin grafting was performed after a good granulation response had been achieved. Currently, all our patients are relapse-free 18 months after completion of heat therapy.

Rifampicin/streptomycin chemotherapy of BU is associated with the development of ectopic lymphoid tissue in the lesions [16]. In some patients, effects reminiscent of the immune reconstitution syndromes observed in tuberculosis and leprosy patients after highly active antiretroviral therapy [17] are observed. In contrast, heat treatment did not lead to massive increases in local inflammation and this less vigorous response may favour rapid re-epithelialization. Also haemorrhages, which are regarded as negative indicators for uncomplicated wound healing [18] were not observed.

Results of two pilot studies, the study of Meyers et al. in the 1970s [10] and our study, demonstrate that heat is a highly efficacious therapy for *M. ulcerans* disease. Use of PCM packs represents a break through for thermotherapy with respect to its practicality in endemic areas with poor infrastructure. Further optimization of the heat treatment schedule should make it suitable for community application.

Acknowledgments

We thank engineer Dr. M. Hellmann for his technical input (Dr. M. Hellmann and TJ together developed the idea of adapting PCM as a heat delivery system to treat Buruli ulcer). We thank the nursing team, Daniel Ze Bekolo, Ekodogo Kombang, Menkaye Samentanga, Serge Ndtoungou, Susan Fese Mboe and Julie Abomo, and Dr. Kemo Hans of the hospital in Ayos, Cameroon, for their outstanding work. We thank S. Braxmeier, Bavarian Center for Applied Energy Research, Functional Materials for Energy Technology, Wuerzburg, Germany, for his technical input in the PCM-based heat application device and Dr. M Kaeser, Department of Medical Parasitology / Infection Biology, Swiss Tropical Institute, Basle, Switzerland, for investigating the samples by real-time PCR.

Figures

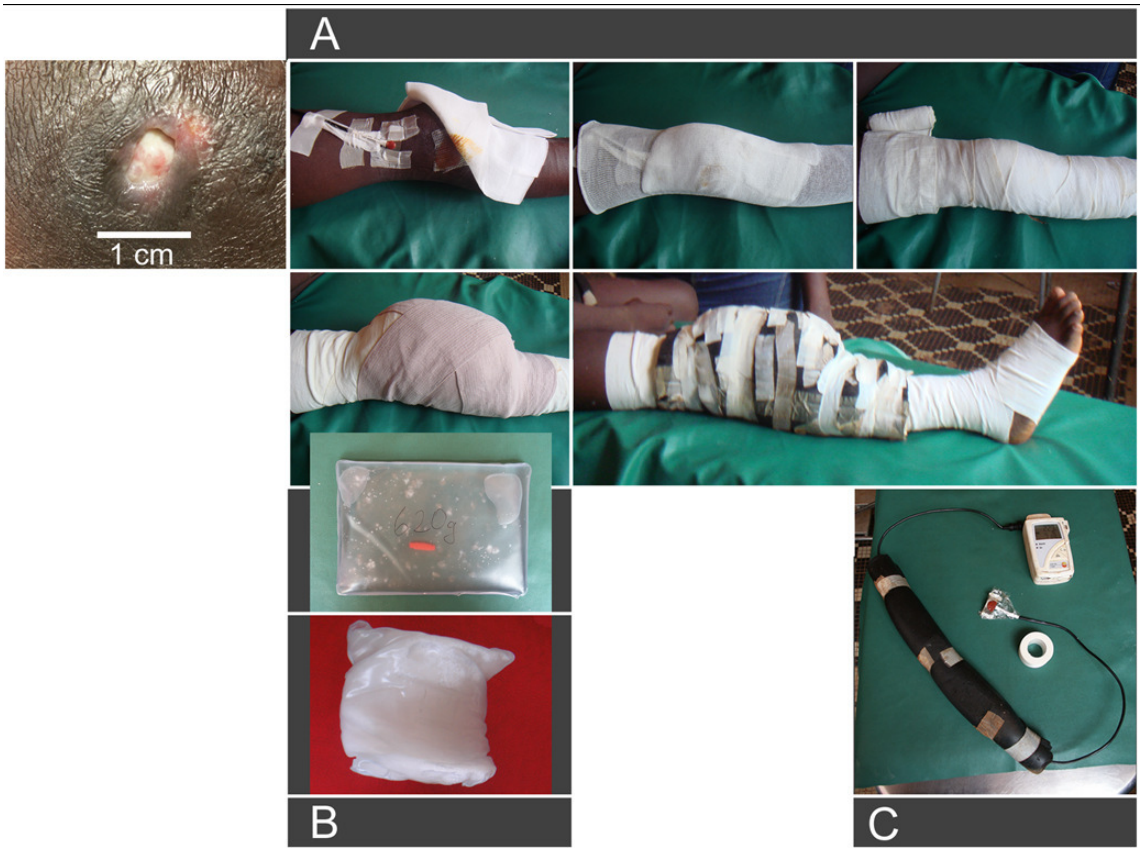


Figure 1. Mounting of the PCM-based heat application system and temperature monitoring device. (A) PCM pack and bandage mounted for treating an ulcer on the lower limb (patient 2) and temperature monitoring system, (B) PCM pack with sodium acetate trihydrate in the fluid phase before initiating the crystallisation process with the starter (red), sodium acetate trihydrate in the solid phase after the stored heat has been discharged, (C) temperature monitoring system with the sensor connected to the data logger to record the temperature at the skin surface as part of the clinical trial documentation. This will not be needed when the device is put into routine use.

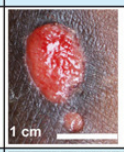
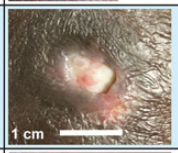
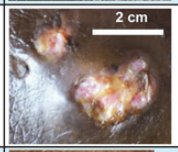
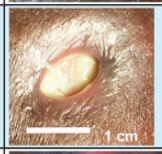
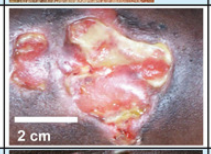
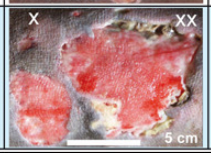
Patient [no age sex]	Ulcer before start of heat treatment	Description of ulcer	Detection of AFB or *M.ulcerans* DNA (swab day 0)	Histopathology (punch day 0)	Heat treatment [T above / for hrs/day **no of days** no of PCM- packs]	Size reduction cm² (%)	
no 1 9 yrs m		Ulcer, left upper arm, no undermined edges, no oedema	Swab neg PCR weak pos	No AFB, some mixed infiltration, no necrosis, some fat cell ghosts, psoriasiform epidermal hyperplasia	≥ 39 °C / 8.4 hrs/day ≥ 40 °C / 5.1 hrs/day ≥ 41 °C / 2.2 hrs/day ≥ 42 °C / 0.4 hrs/day **28 days** 1 PCM-pack	start 1 week 2 weeks 3 weeks 4 weeks	1 (100) 1.1 (110) 0.3 (30) 0.1 (10) 0 (0)
no 2 11 yrs m		Ulcer, right knee, circular undermined edge, cotton wool appearance, little oedema of the margin	Swab neg PCR weak pos	No AFB, acute infiltration (mainly PMNs), slight necrosis, some fat cell ghosts, psoriasiform epidermal hyperplasia	≥ 39 °C / 9.2 hrs/day ≥ 40 °C / 4.7 hrs/day ≥ 41 °C / 1.2 hrs/day ≥ 42 °C / 0.2 hrs/day **31 days** 1 PCM-pack	start 1 week 2 weeks 3 weeks 4 weeks 5 weeks	0.7 (100) 0.9 (129) 0.6 (86) 0.4 (57) 0.3 (43) 0.2 (29)
no 3 11 yrs f		2 ulcers, left forefoot, oedema between ulcers and around large ulcer, no undermined edges	Swab clear pos PCR clear pos	AFB, necrosis and fat cell ghosts, psoriasifrom epidermal hyperplasia, massive mixed infiltration, no granuloma	≥ 39 °C / 10.1 hrs/day ≥ 40 °C / 4.4 hrs/day ≥ 41 °C / 1.3 hrs/day ≥ 42 °C / 0.2 hrs/day **28 days** 1 PCM-pack	start 1 week 2 weeks 3 weeks 4 weeks 5 weeks	2.8 (100) 2.1 (75) 1.4 (50) 0.5 (18) 0.1 (4) 0 (0)
no 4 6 yrs f		Ulcer, left upper arm, circular undermined edge, cotton whole appearance, oedematous margin up to 4 cm, raised max 1 cm above skin level	Swab clear pos PCR strong pos	No AFB, typical massive necrosis and fat cell ghosts, psoriasiform epidermal hyperplasia	≥ 39 °C / 10.9 hrs/day ≥ 40 °C / 5.9 hrs/day ≥ 41 °C / 1.5 hrs/day ≥ 42 °C / 0.2 hrs/day **53 days** 1 PCM-pack	start 1 week 2 weeks 3 weeks 4 weeks 5 weeks	1.1 (100) 1.3 (118) 1.1 (100) 0.7 (64) 0.6 (55) 0.5 (45)
no 5 21 yrs f		2 ulcers, right upper arm, separated by skin bridge, undermined edges at 3, 6 and 9 o'clock, oedema of the margin and the skin bridge	Swab clear pos PCR strong pos	No AFB, large necrotic area, minor leukocyte infiltrates, numerous fat cell ghosts, psoriasiform epidermal hyperplasia	≥ 39 °C / 13.2 hrs/day ≥ 40 °C / 9.3 hrs/day ≥ 41 °C / 4.6 hrs/day ≥ 42 °C / 1.0 hrs/day **50 days** 2 PCM-pack skin graft	start 1 week 2 weeks 3 weeks 4 weeks 5 weeks	12 (100) 13 (108) 13 (108) 14 (117) 13 (108) 12 (100)
No 6 15 yrs f		2 ulcers, right lower leg, separated by skin bridge, undermined edges 9-12 o'clock proximal (x), 1-9 o'clock distat (xx), filled with slough. Induration extending down to the ankle	Swab strong pos PCR strong pos	No AFB, massive necrosis and fat cell ghosts, psoriasiform epidermal hyperplasia	≥ 39 °C / 13.1 hrs/day ≥ 40 °C / 8.0 hrs/day ≥ 41 °C / 4.4 hrs/day ≥ 42 °C / 1.0 hrs/day **55 days** 4 PCM-pack skin graft	start 1 week 2 weeks 3 weeks 5 weeks	27 (100) 43 (159) 43 (159) 43 (159) 38 (141)

Figure 2. Baseline data, heat treatment schedules and results.

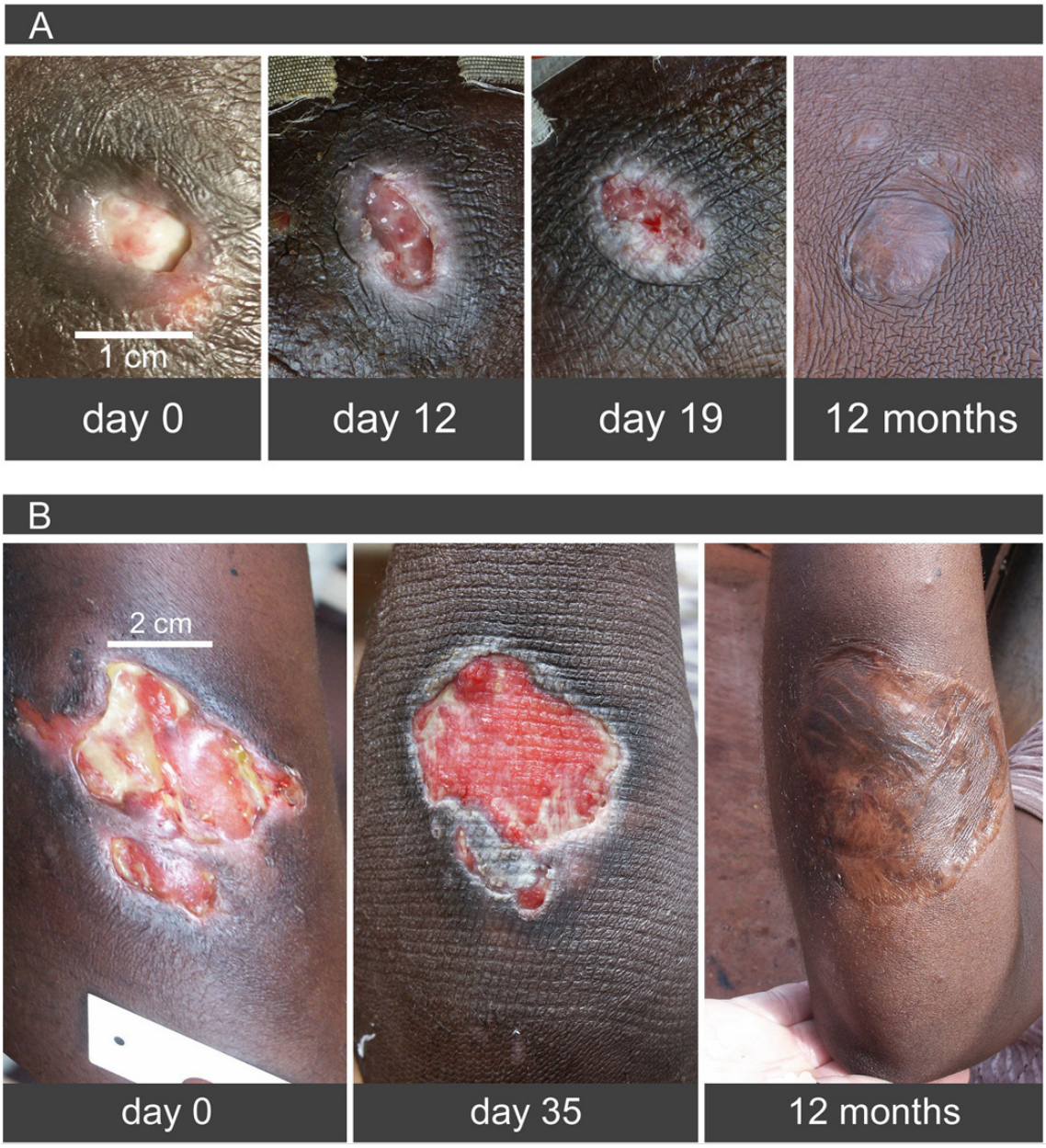


Figure 3. Healing of Buruli ulcers under PCM-based heat treatment and long term results. (A) Patient 2, (B) patient 5: Progress of healing during heat treatment. Note in particular early onset of epithelialisation. Far right follow-up 12 months after completion of heat treatment. Patient 5 (B) after skin grafting.

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CHAPTER 8

Heat treatment of Buruli ulcer is not associated with massive local immune activation

Schütte D [1], Junghanss T [2], Um-Boock A[3], Pluschke G [1]

[1]Dept. Medical Parasitology / Infection Biology, Swiss Tropical Institute, Basle, Switzerland
[2]Section Clinical Tropical Medicine, University Hospital, Heidelberg, Germany
[3]ALES, Bureau Régional pour l'Afrique, Yaoundé, Cameroon

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Abstract

Buruli ulcer is a necrotizing skin disease caused by *Mycobacterium ulcerans*. These bacteria grow best at temperatures of 30 – 33 °C and produce the cytotoxic macrolide toxin mycolactone, to which most of the clinicopathological symptoms are related. To date, daily intake of rifampicin and streptomycin and/or surgical excision of the affected tissue are the standard treatments.

Therapy with local application of temperatures around 40°C using a phase change material-based heat application system is currently under evaluation. Here we have analysed punch biopsies taken at treatment days 0 and 28 to monitor histopathological changes and local immunological reactions during heat therapy. While massive infiltration and development of ectopic lymphoid tissue is observed during antibiotic therapy, the extent of total leukocyte infiltration did not increase during thermotherapy. While the number of PMNs decreased, T cells became more abundant. This may favour a rapid transition from inflammation to healing, as indicated by the clinical response to heat treatment, which was characterised by an extraordinarily rapid re-epithelization and healing process.

Introduction

Buruli ulcer (BU) is a dermatological infection with *Mycobacterium ulcerans* leading to chronic necrotizing skin ulcers. The highest incidence rates are reported from countries of Central and Western Africa with the majority of cases being children [1]. When left untreated the infection spreads locally and can destroy huge parts of the patient's body surface. Treatment with antibiotics and surgery is time-consuming and difficult, because most affected communities are located in remote areas of the developing countries [2]. Spontaneous healing has been reported though is not commonly observed and those individuals usually exhibit tremendous scarring leading to joint contractures and disabilities [3].

M. ulcerans grows best at relatively low temperatures around 30°C and already in the 1970s Meyers et al conducted a small trial on local heat application as treatment option. Results were very promising as patients could be cured without any further surgical therapy required, but the heat application device used was electricity dependent with a complicated thermostat construction [4]. Recently, Junghanss et al performed proof-of-principle trial with six patients with laboratory reconfirmed ulcerative BU lesions in Cameroon with a new low-tech approach of heat application. Plastic bags filled with sodiumacetatetrihydrate, a cheap and non-toxic phase-change material (PCM), were used to maintain a stable temperature of around 40°C at skin level to prevent *M. ulcerans* from proliferation and toxin production (Junghanss et al. 2008, submitted).

The temperature sensitivity of *M. ulcerans* favours development of lesions in the skin and underlying tissue where mycolactone causes progressing dermal necrosis, destruction of infiltrating immune cells and a suppression of local immune responses [5-7]. In active BU lesions, intra-lesional cellular immune responses are usually mild to absent and the vast majority of bacteria are extracellularly clustered in the necrotic subcutaneous tissue [8,9]. Very early lesions or the macroscopically healthy margins of chronic BU lesions may contain a more pronounced leukocyte infiltration and here intracellular bacteria can occasionally be observed [10-12]. In previous studies we could show that antibiotic therapy with rifampicin and streptomycin does not only kills the bacilli but also reverses local immunosuppression. During therapy a massive increase of leukocyte infiltration in the lesions as well as the development of ectopic organised lymphoid aggregates is observed [9].

Here we report on the local cellular immune response in BU lesions during thermotherapy. In patients treated with the PCM-based heat application device the clinical picture was ameliorating extraordinarily rapid. Wound closure was achieved by self-healing in small ulcers or skin grafting in larger lesions (Junghanss et al. 2008, submitted). Our histopathological evaluation indicates that lack of massive local immune responses favour rapid transition from inflammation to tissue healing.

Materials and Methods

Six laboratory-confirmed BU cases were included in this study and admitted to local heat treatment of the affected body parts as has been described (Junghanss et al 2008). Briefly, plastic bags filled with the PCM sodiumacetatetrihydrate were applied to the wounds and a therapeutic temperature of about 40°C was kept at skin surface level for 15-20 hours per day for at least four weeks. Temperature was recorded automatically every 10 min for safety reasons and wounds cleaned and dressed three times a day to avoid additional super-infection. While 4/6 lesions healed completely without any other medical intervention apart from dressing and heat application, 2/6 required additional skin graft to close the large ulcerative areas without further delay to circumvent exaggerated scarring. All individuals remained relapse free within 12 months of follow-up. Biopsies and swabs were taken and assessed for presence of AFB by ZN staining, histopathology, as well as presence of *M. ulcerans* DNA by IS2404 real-time PCR.

4mm punch biopsies were taken at days 0 and 28 from the lesion margins and fixed in 4% neutral-buffered paraformaldehyde for 24h. Tissue samples were stored in 70% ethanol and transferred to Switzerland, where samples were further processed as previously described [9]. Paraffin embedded tissue was cut into 5µm thin sections. After dewaxing and rehydration, sections were stained with haematoxylin/eosin (HE) or Ziehl Neelsen (ZN) for morphological and microbiological assessment, respectively. Immunohistochemistry (IHC) was performed with antibodies against Elastase (polymorphonuclear neutrophils [PMNs]; Dako), CD3 (T lymphocytes; Dako), CD20 (B lymphocytes; Novocastra) and CD14 (macrophages; Novocastra) using Vector® NovaRED™.

Results

Serial sections from punch biopsies taken at days 0 and 28 from the margins of the six ulcerative lesions were stained with HE and ZN. At day 0, the samples taken from the three small ulcers showed only slight signs of necrosis and no AFB were detected. In these lesions the infection and tissue destruction thus had not yet progressed deeply into the tissue adjacent to the border of the ulcers. However, PCR performed on swab material was positive for IS2404 and one out of these three small ulcers was also AFB positive.

The biopsies from two of three patients with larger ulcers exhibited histopathological changes, i.e. extensive necrosis of the adipose tissue in the absence of inflammation, characteristic for undermined edges of BU lesions. In the third patient with a larger lesion necrosis was associated with a much stronger leukocyte infiltration than is commonly found in BU lesions.

Compared to the corresponding sample taken at day 0, the biopsy taken on day 28 showed rather a reduction than an increase in cellular infiltration (Fig.1-c & -d). On day 0 infiltrates consisted primarily of histiocytes with only few lymphocytes or PMNs and most of the cells were apoptotic/necrotic (Fig.1-c). In contrast, on day 28 the infiltration contained in addition to histiocytes a high proportion of T lymphocytes (Fig.1-d). Already without treatment on day 0 giant cells but no granulomas were found in the deeper dermis (Fig.1-e) and the sub-epidermal layer revealed vasculitis and strong infiltration of the papillary dermis (Fig.1-f). After 28 days of heat treatment both the deeper dermis (Fig.1-g) and the sub-epidermal region (Fig.1-h) contained scarring tissue and elevated levels of fibroblasts building new connective tissue. When stained with ZN for acid-fast bacilli only the biopsy from day 0 comprised small clusters of extracellular AFB inside the necrotic tissue whereas no AFB were found in the biopsy from day 28 (Fig.1-i).

The largest ulcer treated within the framework of this study showed signs of super-infection, such as bad odour and greenish necrosis at the time of admission (Fig.2-a). Swabs taken from the undermined edges of the lesion were strongly positive for AFB and revealed heavy superinfection (Fig.2-b). After 28 days, undermined margins had reattached to the underlying tissue (Fig.2-c), the superinfection was eliminated and only a few AFB were left (Fig.2-d).

To monitor changes in the composition of the cellular infiltrates associated with heat treatment we characterised the infiltrating cells in biopsies from two lesions, one small ulcer and one large ulcer. On day 0 the deep dermal necrosis in the biopsy from the small ulcer showed mild mixed infiltration (Fig.3-a) primarily consisting of Elastase+ PMNs (Fig.3-b) and very few CD3+ T lymphocytes (Fig.3-c). On day 28 the deep dermal infiltration was very low (Fig.3-d) but the sub-epidermal area exhibited increased cellular infiltration mainly around vessels (Fig.3-e). Compared to day 0 the density of Elastase+ PMNs was strongly reduced (Fig.3-f), but the infiltrates in the sub-epidermal area comprised CD3+ T lymphocyte clusters (Fig.3-g).

On day 0 the biopsy from the large ulcer showed a minor infiltration in its extended necrotic core (Fig.3-h). Elastase+ PMNs located around the necrotic adipose and connective tissue in the deeper dermis (Fig.3-i) showed often had apoptotic/necrotic appearance (Fig.3-j). CD3+ T lymphocytes were only found around some vessels of the sub-epidermal tissue (Fig.3-k). The biopsy taken on day 28 still contained necrotic areas in the deeper dermal layer and cellular infiltration remained low (Fig.3-l) as were the numbers of Elastase+ PMNs (Fig.3-m). In the upper dermis some CD3+ T lymphocyte foci could be found near glands and vessels (Fig.3-n).

In both lesions, numbers of CD14+ macrophages/monocytes were very low on day 0 and showed only marginal increase on day 28 (not shown). While CD20+ B cells were absent on day 0, some scattered CD20+ cells could be found on day 28 (not shown).

Discussion/Conclusion

Local inflammation of the skin was not evidently altered during thermotherapy, which is in accordance with the clinical observation that lesions did not cause increased pain. Both PMNs, as members of the innate, and T cells, representing adaptive immune response, were present with PMNs mainly located around bacteria containing dermal necrosis and T cells confined to vessels in the upper dermis. Total amounts of infiltrating cells were rather stable or decreasing after four weeks of treatment, leading to the assumption that infection can be cleared without major cellular immune modulations necessary. The relative ratio of PMNs vs. T lymphocytes shifted from almost equal amounts towards a clear T cell dominance over time.

In skin samples from antibiotically treated patients, vast haemorrhages were observed [9] as a negative indicator for uncomplicated wound healing [13] which was not the case during heat application. Furthermore, studies on knock-out mice showed that the depletion of PMNs enhances the re-epithelization of wounds [14]. These data are in concordance with the observed enduring discharge from the ulcers together with a highly efficient healing process of the lesions during heat therapy.

Mycolactone is likely to be the main causative for the typical clinical and histological symptoms observed in Buruli ulcer patients. Although it has been discovered almost a decade ago [5] data about its behaviour inside the wounds including released amount, diffusion kinetics and chemical stability is difficult to gain and still fragmentary. Observational data indicate instability of the unsaturated fatty acid side chain of the toxin and cytotoxic properties are lost within few weeks when stored at 4°C. The question arises whether heat application not only breaks mycolactone production by influencing the bacterial metabolism but even enhances degradation of already released toxin molecules. This could be a possible explanation for the astonishingly quick oedema reduction and re-epithelization of the lesions.

Histopathological results in the present thermo therapy study were in stark contrast to findings from antibiotic treated patients were a counterproductive immunopathology should be considered possible [9]. The fact that punch biopsies are not enough to mirror the total infiltration should be regarded which is a clear interpretation limitation

taking in account the great heterogeneity usually observed between different areas within a lesion [9,15,16]. Since all patients showed similarities in both histopathological changes and clinical presentation this might be an indication for heat application being a potent tool to cure BU lesions without major tissue damage. In combination with antibiotic therapy it could prevent disproportionate local leukocyte reactions and redundantise additional surgical interventions.

Figures

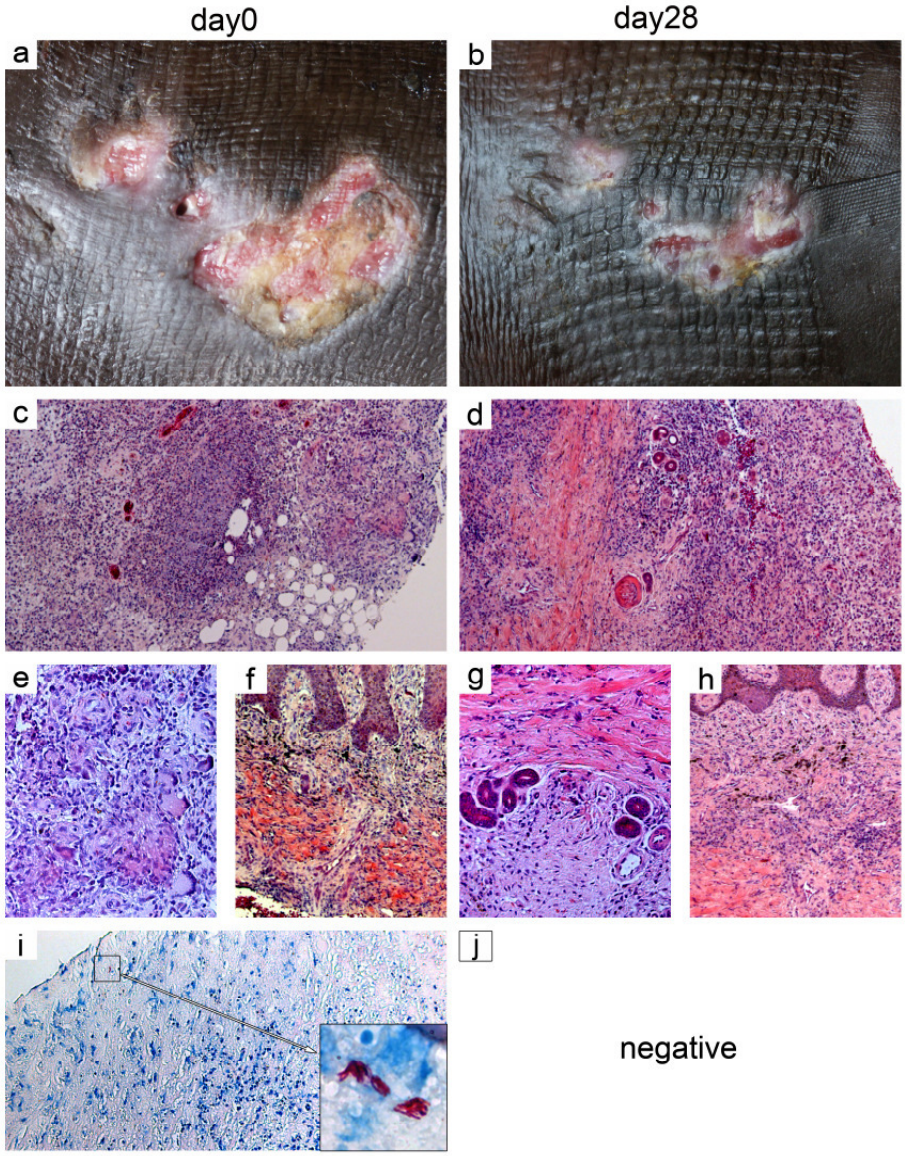

Figure1: Patient with larger ulceration reveals uncommonly strong cellular infiltration

(a-b) Clinical progress during four weeks of heat treatment. (c-h) HE and (i-j) ZN staining of 5mm sections from punch biopsies taken on days 0 and 28.

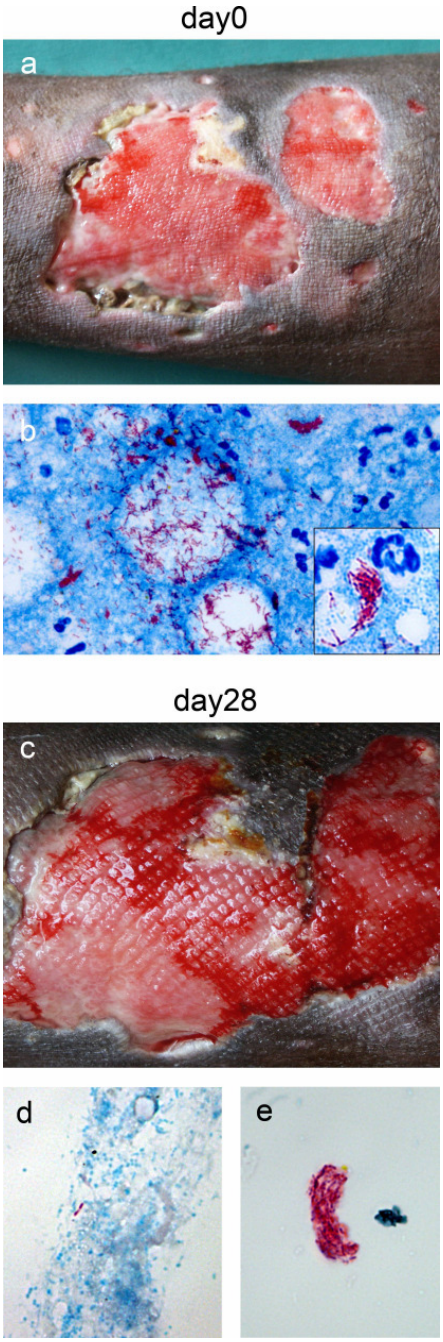

Figure2: Largest ulcer enrolled in the study exhibits rapid improvement during thermotherapy despite heavy superinfection

(a, c) Clinical picture at days 0 and 28. (b, d, e) ZN staining of smears taken from the undermined edges show obvious reduction in bacterial load in the course of heat application.

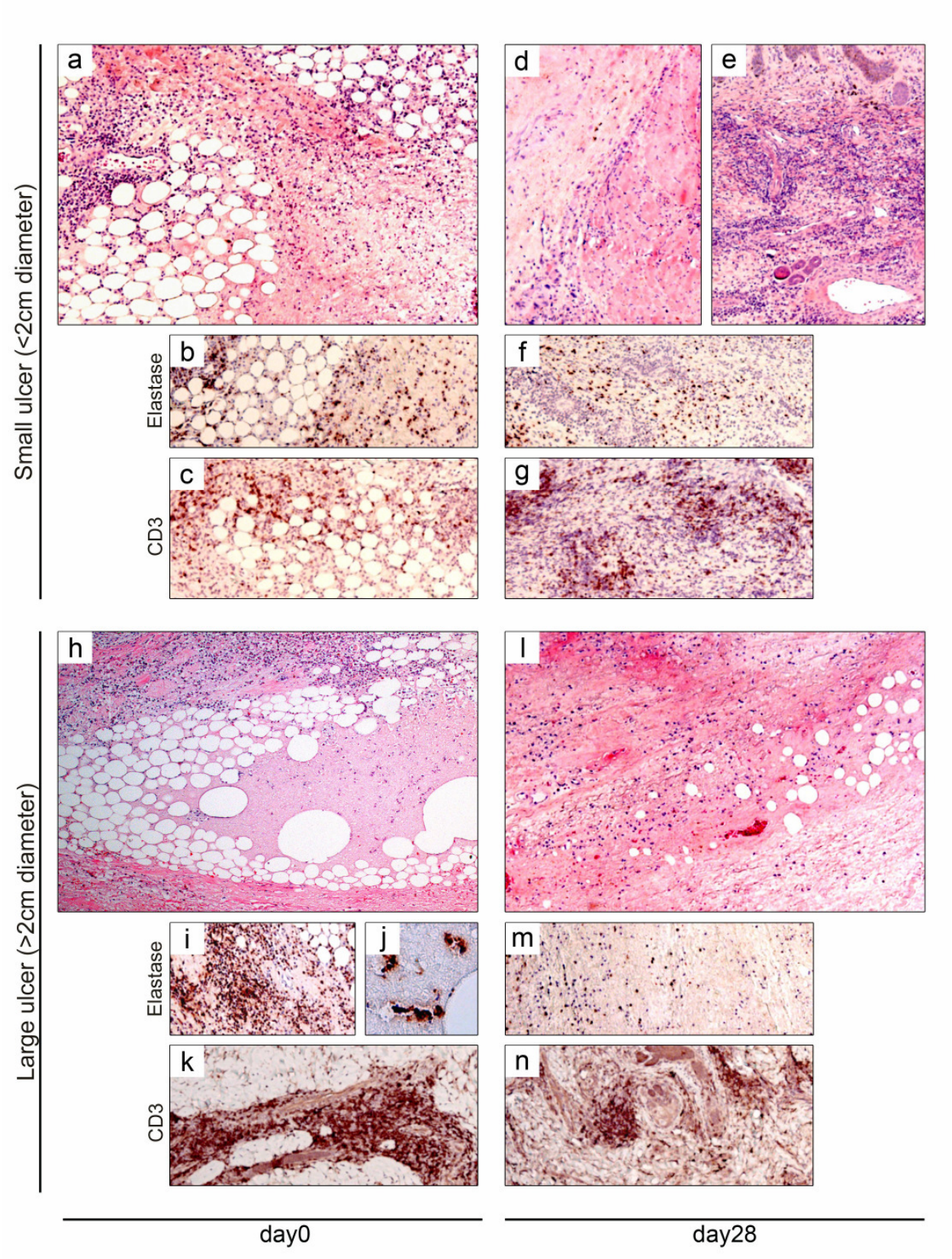
Small ulcer (<2cm diameter)
Large ulcer (>2cm diameter)
Elastase
CD3
a
b
c
d
e
f
g
h
i
j
k
l
m
n
day0
day28

Figure3: Local leukocyte infiltrations diminish during heat treatment

Histological examination of punch biopsies taken from (a-g) a small and (h-n) a larger ulcerative lesion. (a, d, e, h, l) HE staining of sections from days 0 and 28. Immunohistochemistry was performed with antibodies against (b, f, I, j, m) neutrophilic elastase and (c, g, k, n) T lymphocyte marker CD3.

(a-c) Deeper dermis exhibits mild infiltration primarily consisting of elastase+ PMNs and few CD3+ T lymphocytes. (d-g) After four weeks of thermotherapy infiltrates are mainly located around vessels in the upper dermal layer and cellular subsets reveal a switch from acute (elastase+) to chronic (CD3+) infiltrates.

(h-k) Focal infiltration of tissue surrounding the sub-epidermal necrosis consists mainly of elastase+ PMNs. CD3+ T lymphocytes are located around vessels. (l-n) On day 28 overall infiltration has dropped significantly. Only scattered elastase+ PMNs were observed, but the upper dermis still contains CD3+ T cell clusters.

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CHAPTER 9

Compound DS-1 effectively inhibits growth of in vitro cultured Mycobacterium ulcerans

Daniela Schütte, Gerd Pluschke

Medical Parasitology / Infection Biology, Swiss Tropical Institute, Basle, Switzerland

This article is in preparation

Mycobacterium ulcerans, a human pathogen provoking skin ulceration due to its exotoxin mycolactone [1], causes one of the most prevalent mycobacteriosis worldwide (Buruli ulcer). Infected individuals suffer from progressing dermal lesions which frequently lead to disabilities as a result of extensive scarring and joint contractures. Currently recommended therapy includes circumstantial surgical excision of the affected tissue and/or eight weeks daily administration of rifampicin and streptomycin [2].

Surgical treatment is very expensive and time-consuming and needs a well equipped and maintained infrastructure. Streptomycin has to be injected daily into the muscles, which is not very well tolerated by superstitious African populations and causes additional suffering and risks, especially for small children who are most often affected [3]. Furthermore, both antibiotics are front-line drugs for TB treatment and major concerns arise regarding resistant *M. tuberculosis* strains developing in co-infected patients due to the relatively short therapy duration.

Novel therapeutic approaches or drugs are therefore urgently needed and are currently under investigation [4,5]. We tested the *in vitro* anti-mycobacterial activity of compound DS-1 in liquid *M. ulcerans* cultures. Details of the compound such as synthesis or structure can not be disclosed to date due to patent issues. The compound was active against *M. tuberculosis* in previous studies.

20 BacTAlert MB Blood Culture flasks supplemented with 1ml MB/BacT Enrichment Fluid and 1.5ml MB/BacT Antibiotic Supplement each were inoculated with *M. ulcerans* strain ifik-1066089 and incubated at 32°C for 2-3 weeks. When exponential growth phase was reached cultures were harvested and bacterial pellets retrieved by centrifugation for 15 min. After washing with PBS pellets were pooled and resuspended in Middlebrook 7H9-T freezing medium by vortexing aliquots containing sterile glass beads. Suspensions were calibrated to OD600 1, aliquoted and stored at -80°C until further use.

Growth inhibition was measured using Microplate Alamar blue assay (MABA) in a 96 well plate format [6]. An appropriate amount of stock solution was diluted 1:4 in Middlebrook 7H9 complete medium (containing glycerol, Tween and OADC/ADS). 200µl of sterile deionised water was added to the outer-perimeter wells of the 96 well plates and the remaining wells were filled with 100µl 7H9GC broth. 100µl of 4x drug solutions were serially diluted in duplicates using a multi-channel pipette starting from

column 2 and 100µl excess medium was discarded from the wells in column 10. Column 11 was used for negative and positive growth controls. 100µl prediluted bacterial culture was added to all wells except the negative growth controls. Plates were incubated at 28-30℃ for 6 days without CO2 supply under non-rocking conditions. 50 µl of freshly prepared 1:1 mixture of 10XAlamar Blue and 10% Tween 80 was provided to all wells and plates incubated for another 4-6 days depending on intensity of colour change. A digital camera was used to document results and plates evaluated based on the observed colour change. Three independent experiments were performed for each starting dilution.

Several batches of rifampicin and streptomycin were tried as positive control in this assay, but the *M. ulcerans* strain used here was not susceptible to these drugs for unknown reason. The strain was very well adapted to BacTec, indeed keeping its ability to produce mycolactone but passing into log phase within 2 weeks after inoculation.

Compound DS-1 had a MIC_{90} of 0.016 – 0.025 µg/ml or 4.6 – 7.4 µmol/ml for *M. ulcerans* strain ifik9066089 in 7H9-GT medium. Coloric changes of the indicator dye Alamar blue were not as clear-cut as observed with faster growing mycobacteria [7]. This is mainly due to the fact that replication of *M. ulcerans* takes much longer from few days up to several weeks and therefore metabolic activity is lower. Although proliferation of ifik9066089 could be accelerated through adaptation to BacTec medium over a long period, the change to 7H9-GT medium obviously slowed it down again.

Cytotoxicity assays both in cell culture and animal model as well as bioavailability studies have to be performed to evaluate whether compound DS-1 or its derivatives are suitable candidates for antimycobacterial drug development. Furthermore, the MIC_{90} of a range of other mycobacteria will be tested.

The here presented data disclose that compound DS-1 is stable in liquid mycobacterial culture over weeks without loss of its antibacterial activity, very efficiently inhibiting growth of *M. ulcerans*. The main goal is to obtain a save drug which is orally administered, acting rapidly and can be produced at low costs. Compound DS-1 may represent a suitable lead structure for the development of a novel antimycobacterial drug to treat Buruli ulcer patients.

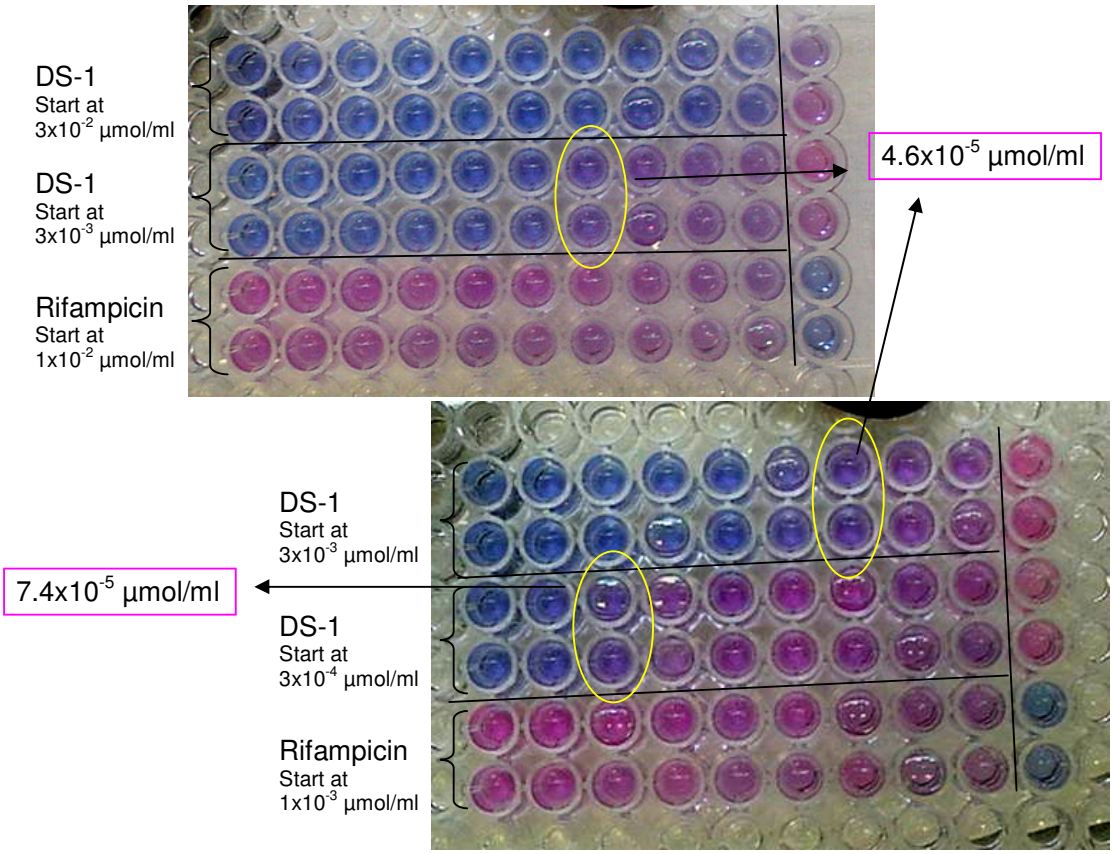

Figure1. MIC_{90} of DS-1 in µmol/ml

MABA with liquid *M. ulcerans* culture on 96 well plates using duplicates of serial 2-fold dilutions of compound DS-1. Last row represents positive (*M. ulcerans* without any drug) and negative (only medium) growth controls. Blue indicates growth inhibition, pink proliferation.

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CHAPTER 10

Diagnosis of *Mycobacterium ulcerans* infection by its 18kD small heat shock surface protein

Daniela Schütte[1], Diana Díaz-Arévalo[1]*, Thomas Bodmer[2], Gerd Pluschke[1]

[1]Swiss Tropical Institute, Section Molecular Immunology, Basle, Switzerland

*Current address: City of Hope, Duarte, CA, USA

[2]University Hospital Bern, Institute for Infectious Diseases, Bern, Switzerland

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Introduction

Buruli ulcer is a chronic necrotizing disease of skin and soft tissue caused by *Mycobacterium ulcerans*. The disease starts as a subcutaneous nodule, papule, or plaque that eventually ulcerates and progresses over months to years [1]. After tuberculosis and leprosy, Buruli ulcer is the third most common mycobacterial infection in immunocompetent humans. People of all ages, races, and socioeconomic class are susceptible, but the main burden of disease falls on children living in sub-Saharan Africa [2]. Buruli ulcer has mainly been reported in countries with tropical and subtropical climates, but it may also occur in some regions where it has not yet been recognized. The full extent of the Buruli disease burden is unknown because this disease occurs primarily in remote and rural areas. The numbers of cases reported in some countries of West Africa are substantial.

Although not generally a fatal condition, BU lesions can become extensive. When diagnosis and treatment are delayed, sufferers are frequently left with long-term physical and cosmetic disabilities. In a known area of endemicity, an experienced person can diagnose BU on clinical grounds. Any two of the following findings are required to confirm clinical diagnosis: (i) acid-fast bacilli (AFB) in a smear stained with Ziehl-Neelsen (ZN), (ii) culture of *M. ulcerans*, (iii) pathognomonic histopathology (contiguous coagulation necrosis and the presence of AFB), or (iv) the presence of *M. ulcerans* DNA as detected by PCR [3]. Culture confirmation may take 8 to 12 weeks and treatment needs to be initiated much sooner than this to ensure an optimal outcome for the patient. The use of PCR for diagnosis of BU has been a major step forward, most commonly targeting IS*2404*, a multicopy transposase [4]. However, this technique has to be performed in a laboratory with high standards to avoid false positives. Histopathology is a reliable diagnostic tool, but certainly a technically demanding method. Among the tests recommended for the confirmation of a suspected case of Buruli ulcer (BU), only microscopy is simple, less costly and within the competence of peripheral treatment centres [5].

Broad antigenic cross-reactivity between mycobacterial species represents a major problem for the development of new tests to identify *M. ulcerans* with high specificity and sensitivity. In a previous work we identified the highly immunogenic 18kD small heat shock protein (shsp) of *M. ulcerans*, which has no homologues in *M. bovis* and

M. tuberculosis, and described humoral responses of individuals exposed to *M. ulcerans* on the basis of this antigen [6]. We produced monoclonal antibodies (mAbs) against this particular protein which recognize *M. ulcerans* antigen preparation in Western Blot and may become useful in the diagnosis of BU.

Here we present analysis of mAbs against *M. ulcerans* 18kD shsp regarding their suitability to monitor *M. ulcerans* infection or exposure. We could show that these mAbs detect *M. ulcerans* in culture and tissue with high sensitivity and specificity and may be used in future for the development of an antigen capture assay to rapidly diagnose *M. ulcerans* infection.

Results

Expression of 18kD shsp under different growth conditions

We have evaluated the potential of a monoclonal anti-18kD antibody with very limited cross-reactivity with other mycobacterial species, namely *M. chelonae* [6] to detect *M. ulcerans* inside BU lesions.

First, we assessed the expression of 18kD shsp *in vitro* under different growth conditions mimicking the change between environment and host. *M. ulcerans* were grown on LJ-medium at 28°C or 32°C under aerobe and at 32°C under anaerobe conditions, and protein lysates loaded onto a SDS-Gel. Mycobacterial proteins were retrieved by beat-beating with 1% SDS solution and another beat-beating of the solid phase with 5% SDS solution. Ponceau staining showed similar protein levels for all lanes (Fig.1a). In the 1% SDS fraction 18kD protein could only be detected under 32°C anaerobic conditions, but the 5% SDS fraction revealed also a certain expression in the 28°C and 32°C culture (Fig.1b). Taken together 18kD seems to be constitutively expressed, but expression levels may vary with growth conditions.

Evaluation of different staining techniques to detect *in vitro* grown *M. ulcerans* with a monoclonal anti-18kD antibody (DD3.6)

We performed immunohistochemistry with the monoclonal anti-18kD antibody DD3.6 on two different African *M. ulcerans* strains, namely ifik and afr2, from culture grown at 32°C on LJ-medium and several negative controls. Both *M. ulcerans* cultures showed positive staining (Fig.2a) whereas other mycobacterial species, namely *M. marinum*, *M. tuberculosis* and *M. bovis,* remained negative (Fig.2b). The ifik strain was used to compare different staining methods for sensitivity and specificity. Peroxidase-based staining with Vector NovaRed using a horse anti-mouse secondary antibody revealed bacterial cluster with blebbing of 18kD containing matrix (Fig.2c; left panel). Single bacteria sprouting from huge culture clumps were easily identified (Fig.2c; right panel). Fluorescence staining with a goat anti-mouse secondary antibody labelled with fluorofore Alexa488 gave a bright signal at lower magnifications (Fig.2d; left panel) and clearly labelled the membrane-bound 18kD shsp (Fig.2d; right panel). Staining with FastRed using a horse anti-mouse secondary antibody labelled with alkaline phosphatase gave a brighter signal compared to (c) and (d) both in bright field and fluorescence microscopy (Fig.2e; left panel). At higher

magnification a high background noise was observed and no clear staining of bacilli could be obtained (Fig.2e; right panel). Secondary antibodies were all manufactured by Vector Laboratories.

To assess the antibody's potential as diagnostic tool we used different immunohistochemistry techniques to detect *M. ulcerans* in skin tissue from BU patients. Staining with Vector NovaRed gave a bright, background-free signal (Fig.3a-i). Higher magnification revealed a very distinct staining of the surface-bound 18kD shsp identifying single organisms within clusters (Fig.3a-ii). Polar accumulation of the protein was found in a proportion of bacteria which showed signs of decomposition (Fig.3a-iii). With Alexa488 fluorescence staining bacteria were efficiently labelled but auto-fluorescent erythrocytes (arrow) inside the tissue interfered with the bacterial staining and reduced sensitivity (Fig.3b-i). The close-up displayed equally sensitive surface staining of 18kD shsp as with Vector NovaRed (Fig.3b-ii). The brightest staining both in bright field and fluorescence was obtained with the phosphatase-based FastRed staining (Fig.3c-i). A spotted staining pattern was observed not allowing identification of single bacteria (Fig.3c-ii). Furthermore, false positive background staining of tissue compartments was produced (not shown).

Discussion

As diagnosis of BU is usually based on clinical examination and unspecific staining of acid-fast bacilli, developing a rapid diagnostic test to identify *M. ulcerans* infections with high specificity and sensitivity is a prioritized topic in BU research. In the present work we evaluated the immunodominant 18kD shsp of *M. ulcerans* and a monoclonal antibody against this antigen for their potential to be used as diagnostic tools.

The environment harbours unknown mycobacterial species that might facilitate serum antibody production in humans and therefore contribute as well to false positive results based on cross-reactive serum antibodies. Diagnosis of BU based on specific monoclonal antibodies against the native *M. ulcerans* 18kD shsp would circumvent those issues. In a former publication of our group monoclonal antibodies against this protein was raised in mice and showed no cross-reactivity to other mycobacterial species except *M. chelonae* in Western Blot analysis [6]. We could reconfirm these results with IHC of different mycobacterial cultures.

It has been shown that *M. ulcerans* grows more efficient and rapid under low oxygen concentration [7]. Moreover, expression of heat shock proteins is commonly up-regulated under hypoxic conditions or due to elevated temperature [8]. Accordingly, 18kD shsp expression seems to be up-regulated when culture conditions were changed from 28°C aerobic towards 32°C anaerobic. We speculate that these findings might reflect the natural situation, where the bacteria have to adjust their metabolism both to an environmental reservoir (low temperature, high oxygen) as well as to a human or animal host (elevated temperature, reduced oxygen). Increased resolution and staining intensity inside BU tissue samples compared to bacteria cultured on LJ-plates further supports this hypothesis.

Fluorescence staining gives a very good contrast for identification of single bacteria but the complex organization of the skin, which carries auto-fluorescent structures such as erythrocytes, confuses the readout and may lead to false positivity. The brightest staining assessable in both bright field and fluorescence microscopy can be achieved with phosphatase-based FastRed staining, but specificity is very low as also nuclei and other structures become lightly stained. The best working procedure is peroxidase-avidin/streptavidin-based immunohistochemistry with the substrate Vector NovaRed, which can be assessed in bright field microscopy and provides high specificity and sensitivity without background noise. Morphology of single *M. ulcerans*

inside and around bacterial clusters can be visualized with this technique. Furthermore, decomposing bacilli developing polar accumulation of 18kD can be observed. This is probably due to loss of cytoplasm and subsequent collapse of the cell wall which results in the “beaded” appearance of bacteria.

The anti-18kD antibody DD3.6 specifically identifies *M. ulcerans* in culture as well as in tissue and we will assess the possibility to determine viability of *M. ulcerans* via this method. Furthermore, a rapid diagnostic test suitable for sample analysis in the field may be developed using monoclonal antibodies against this protein.

Figures

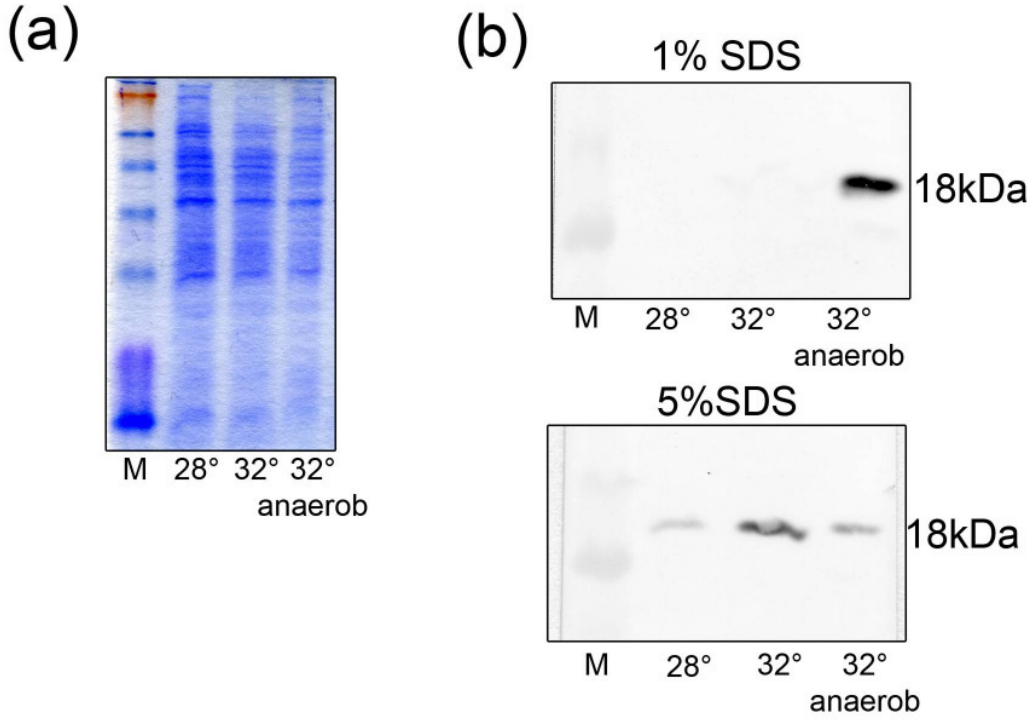


Figure1. Western blot analysis of protein lysates of *M. ulcerans* grown under different conditions

Proteins were extracted in two fractions using first 1% SDS and subsequently 5% SDS on the bacterial pellets retrieved from the first fraction. (a) Ponceau staining showed that equal amounts of protein were loaded onto the gel. (b) Expression of 18kD shsp is up-regulated due to increased temperature and anaerobic conditions.

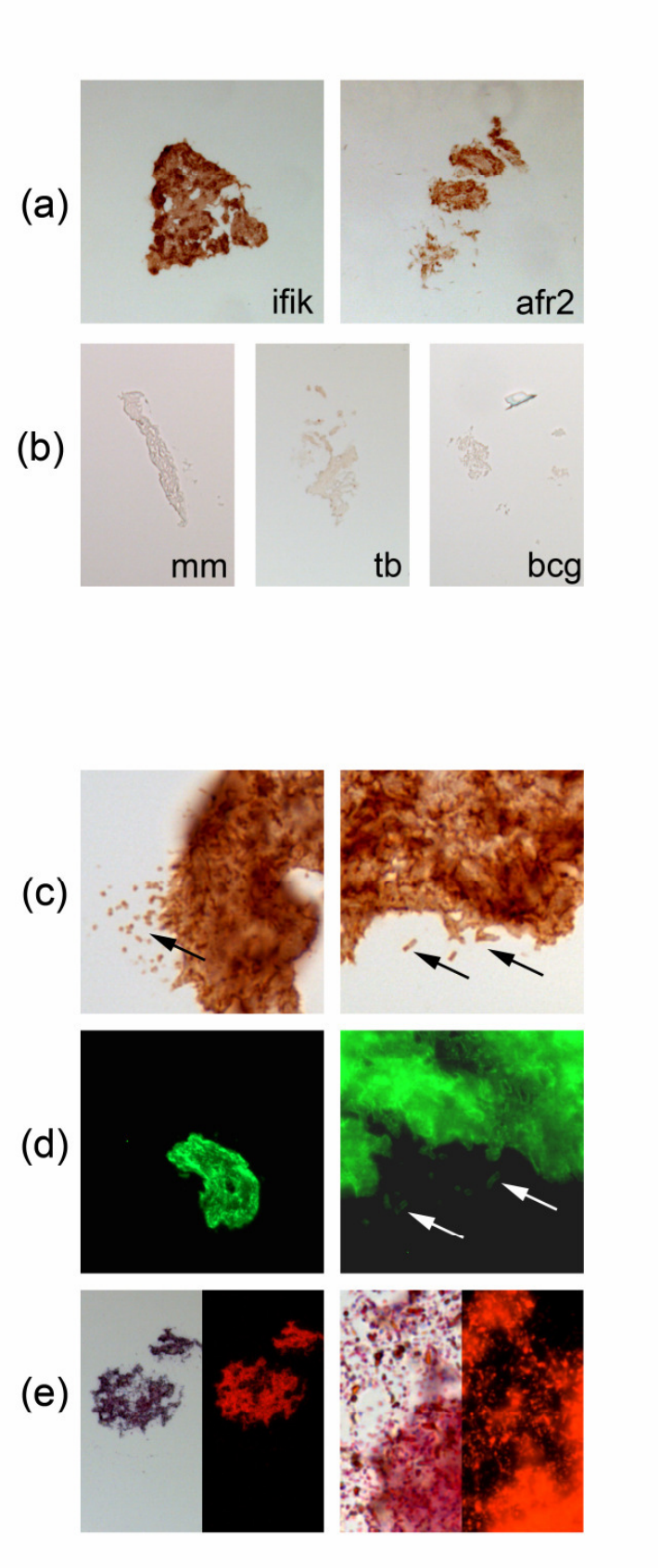

(a)
ifik
afr2
(b)
mm
tb
bcg
(c)
(d)
(e)

Figure2. Staining of cultured mycobacteria using monoclonal antibodies against 18kD shsp

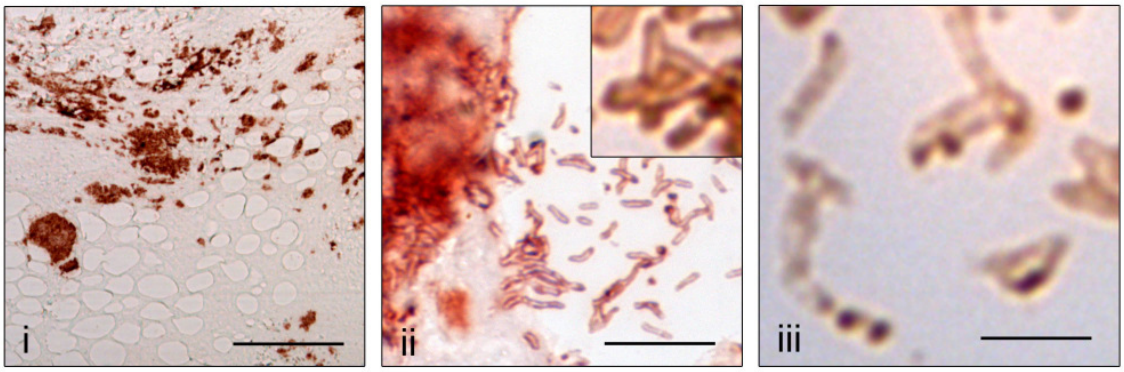
(a) Staining of two different African *M. ulcerans* strains with antibody DD3.6 using Vector NovaRed. (b) No cross-reactivity with *M. marinum*, *M. tuberculosis* or *M. bovis*. (c) Close-up of (a) reveals blebbing of matrix containing 18kD (left panel) and identifies single bacilli (right panel). (d) Fluorescence staining of 18kD shsp with Alexa 488 gives sufficient intensity (left panel) and marks the surface of single bacteria (right panel). (e) Staining with Vector FastRed can be detected with bright field as well as fluorescence microscopy (left panel) but resolution is lower compared to (c) and (d) (right panel).

Figure3. Detection of *M. ulcerans* in human tissue samples with monoclonal antibodies against 18kD shsp using different staining procedures

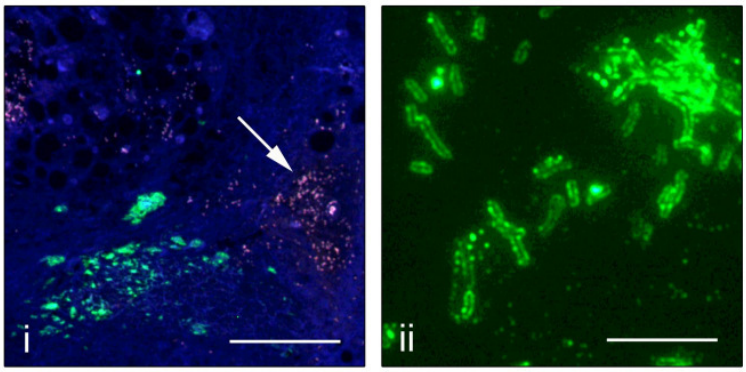
Immunohistochemistry using peroxidase-based Vector NovaRed (a), Alexa488 fluorofore (b) or phosphatase-based FastRed (c). Vector NovaRed produces a fully background-free staining of bacterial clusters (a-i). Higher magnification reveals surface staining pattern (a-ii). Distinct bacterial morphology can be visualized (a-iii) showing polar accumulation of 18kD protein in decomposing *M. ulcerans* (a-iv). Staining of bacteria with Alexa488 can be easily detected at low magnifications, but erythrocytes reveal strong auto-fluorescence (arrow) (b-i). As in (a), surface staining (b-ii) can be illustrated. Bright field and fluorescence images of *M. ulcerans* stained with FastRed (c-i). Higher magnification reveals a spotted, non-distinct staining pattern, but a very strong fluorescence signal (c-ii).

Scale bars: 200µm (a-i; b-i), 50µm (c-i), 16µm (c-ii), 10µm (a-ii), 6µm (b-ii), 4µm (a-iii).

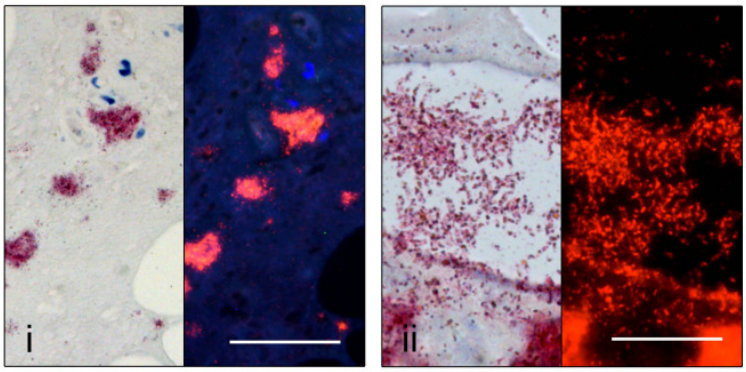
(a)



(b)



(c)



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CHAPTER 11

Immunosuppression and treatment-associated inflammatory response in patients with Buruli ulcer

Daniela Schütte (first author)
Biologist, PhD student
Swiss Tropical Institute, Molecular Immunology, Basle, Switzerland

Prof Dr Gerd Pluschke (corresponding author)
Head of MPI (Medical Parasitology and Infection Biology)
Group leader Molecular Immunology
Swiss Tropical Institute, Molecular Immunology, Basle, Switzerland

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Abstract

Buruli ulcer is a necrotizing skin disease caused by *Mycobacterium ulcerans*. Major necrosis with abundant clusters of extracellularly replicating mycobacteria and only minor leukocyte infiltration are characteristic histopathological features of the disease. Mycolactone, a cytotoxic macrolide exotoxin of *M. ulcerans* plays a key role in the development of this pathology. Antimicrobial therapy, such as rifampicin/streptomycin that was recently introduced, appears to lead to phagocytosis of mycobacteria and massive leukocyte infiltration which culminates in the development of ectopic lymphoid structures in the lesions. While the curative effect of the antibiotic treatment may be supported by immune defense mechanisms, persisting mycobacterial antigens and immunostimulators occasionally also seem to cause apparent reactivation of the disease. This appears to be related to excessive immunostimulation rather than incomplete killing of the pathogen.

Introduction

Epidemiology of BU

First reports about extending cutaneous ulcers in the Ugandan population came from Sir Albert Cook in 1897 indicating that BU was already present at that time in Africa [1]. In 1937 the disease was first recognized in Bairnsdale in Victoria, Australia [2] and in 1948 McCallum et al were able to culture and identify *Mycobacterium ulcerans* as the causative agent of the disease [3]. Nowadays, the so-called Bairnsdale or Buruli ulcer (named after the Buruli County of Uganda, where a BU epidemic occurred in the 1960s) is the third most common mycobacterial infection in immunocompetent people [4]. BU has been found by now in more than 30 tropical and subtropical countries worldwide [5]. Endemic foci are usually linked to wetlands and riverine areas, and especially in many regions of West-Africa including Benin, Côte d'Ivoire, Cameroon and Ghana the numbers of reported BU cases have dramatically increased in recent years [6,7,8]. Outbreaks appear to be related to environmental changes and an increased exposure of affected populations [9,10]. Although the disease is known to develop in all age groups with a nearly equal gender distribution, children aged between 5 and 15 make up the majority of patients

[11,12]. Occasional clustering of cases within one family may reflect a common source of infection or increased genetic susceptibility to infection rather than human-to-human transmission [13].

It is commonly believed that *M. ulcerans* is an environmental mycobacterium and that micro-traumata of the skin during fishing, farming or washing activities represent entry-sites for infection [14]. However, it is not clear, whether the natural reservoir relevant for *M. ulcerans* infection in humans has already been identified. Aquatic plants are known to carry bio-film formations of *M. ulcerans* [15] and several groups of aquatic animals, such as aquatic insects, mollusks, frogs and fish have been found associated with *M. ulcerans*. Recently, the first successful isolation of *M. ulcerans* from an environmental source, a water strider collected in West-Africa, has been published [16]. Insects such as mosquitoes and biting aquatic hemiptera have been implicated as vectors [17,18], but positivity for *M. ulcerans* may only be an indicator for the presence of the pathogen in the environment and not for a role of the insects in transmission [19,20]. Ulcerative lesions caused by *M. ulcerans* have been described not only in humans, but also in wild and domestic animals [21,22,23].

Clinical presentation of BU

BU is primarily an infection of the subcutaneous adipose tissue. Most commonly it starts as a painless, movable subcutaneous nodule or papule adherent to the skin (Figure1). The painless nature of progressive BU seems to be due to nerve damage at the site of the lesions [24]. All parts of the body can be affected, but most lesions are located at the extremities. Thermosensitivity of *M. ulcerans* [25] seems to favor development of skin lesions of the limbs. Early lesions may either heal spontaneously or break down centrally and form a necrotic skin ulcer. Other manifestations of early disease are large plaques or edema, which often extend rapidly, eventually leading to large ulcerations [26,27]. Usually the ulcers have deeply undermined edges and enlarge over an extended period of time (Figure 1). They are often painless, systemic symptoms are largely absent and involvement of visceral organs has not been described [26]. After destruction of skin and soft tissue, reactive osteitis or osteomyelitis may develop [28]. A subset of patients develops multiple skin lesions, and lymphohematogenous spread of the mycobacteria may also lead to metastatic osteomyelitis [27]. After extended periods of progressive ulceration, the necrotic

stage of the disease may cease and lesions may heal spontaneously. Scarring close to joints often result in contractures and severe functional limitations [29]. Since *M. ulcerans* isolates from the same geographical origin are genetically largely monomorphic [30,31], variations in disease progression and severity observed within a particular endemic area are most likely related to host factors.

Treatment of BU

Until recently, surgical excision of lesions with a wide margin including healthy appearing tissue was the only recommended treatment for all stages of BU [11,12,21]. While for pre-ulcerative lesions primary closure after wide excision is possible, split skin autografting after development of granulation tissue is required for larger lesions. Recurrence rates between 6% to 47% have been reported after surgical treatment [32,33,34] and adjunct antibiotic therapy has therefore sometimes been implemented [35]. Recurrences may be caused by small numbers of *M. ulcerans* that have spread to non-excised macroscopically healthy tissue surrounding the primary lesion [36].

Since 2004 WHO recommends a dual antibiotic therapy with oral rifampicin (10 mg/kg) and i. m. streptomycin (15 mg/kg) administered daily for at least eight weeks [37]. For nodular lesions it has been shown that culture turns negative after four weeks of this chemotherapy [38]. Results of a first published observational study are encouraging [39]. Of 215 treated patients, 102 were treated exclusively with antibiotics. 113 had to be treated with antibiotics plus surgical excision and skin grafting. One year after antibiotic treatment was completed, only 3 of the 208 retrieved patients had a recurrence [39]. Since both streptomycin and rifampicin treatment can be associated with side effects [40], search for alternatives, in particular for a fully oral drug treatment is ongoing. Even if showing reasonable antimicrobial activity *in vitro*, some potential alternative drugs such as macrolides and fluoroquinolones, had no satisfactory curative effect in experimental animal models for BU [41,42,43]. In contrast, moxifloxacin and clarithromycin, have shown bactericidal activity in mice [44]. Other topical treatments including application of heat [45], mineral clay [46], nitrite ointment [47], hyperbaric oxygen [48], and even honey [49] have been suggested.

Virulence of *M. ulcerans*

Evolutionary origin of M. ulcerans

Comparative genomic analyses have shown that *M. ulcerans* has developed from a progenitor of the aquatic environmental mycobacterium *M. marinum* [30,31,50]. In contrast to *M. ulcerans*, *M. marinum* only causes self-limiting granulomatous infections in humans. Acquisition of the virulence plasmid pMUM001, encoding polyketide synthases that produce the macrolide cytotoxin mycolactone [30,31,51], massive gene decay (loss of 1.1 Mb of genomic DNA) and pseudogene formation [50] are hallmarks of the development of *M. ulcerans* into a pathogen that causes chronic infections. Reductive evolution indicates that *M. ulcerans* is adapting to a new niche environment. Gain of the immunosuppressive toxin mycolactone is accompanied by loss of highly immunogenic proteins [52], suggesting an adaptation to survival in host environments that are screened by immunological defense mechanisms. Comparative genomic analyses indicate that *M. ulcerans* has developed two distinct lineages: (i) the “classical” lineage representing the most pathogenic genotypes – those that are found in Africa, Australia and South East Asia; and (ii) an “ancestral” lineage comprising strains from China/Japan, South America and Mexico [30,31].

Mycolactone structure and activity

One key to the understanding of the pathology of *M. ulcerans* infection is the family of macrolide exotoxins designated mycolactones. To date, the structure of six different forms of mycolactone has been elucidated. These consist of a common lactone core to which an unsaturated fatty acyl side chain is appended (Figure 2) [53,54]. Variations in length and oxidation state of this side chain defines the different forms, i.e. mycolactone A to D produced by *M. ulcerans* lineages isolated from different regions [55,56,57], mycolactone E coming from *M. liflandii* [58] and mycolactone F recently discovered in *M. pseudoshottsii* [59]. Mycolactones lead to an arrest in the G0/G1 of the cell cycle followed by apoptosis [53]. The type(s) of mycolactone produced by a certain *M. ulcerans* lineage may have major implications for its virulence. In-vitro, mycolactone C for instance is 10,000-fold less active than A and B [57].

In vitro activities of mycolactone

The molecular mechanism of mycolactone action on eukaryotic cells is not entirely clear, but current evidence indicates that it diffuses through the plasma membrane, accumulates in the cytoplasm and interacts with a cytosolic target [60]. Within a short time, mycolactone can cause dramatic cell skeletal rearrangements, which may the cause for reduced phagocytic activity of mycolactone-treated macrophages [61,62,63]. The cytotoxic action of mycolactone is not specifically directed towards a certain cell type, but rather affects a broad range of cell types although in differing intensity, which is also dependent on the state of maturation of the target cells [54,64]. Adipose cells are for instance extremely sensitive to mycolactone and undergo apoptosis at low toxin concentrations, whereas other cell types cope better [65]. This is in accordance with the histopathological observation of extensive adipose coagulative necrosis below a relatively intact sub-epidermal and epidermal layer in early BU lesions.

In vitro studies have identified multiple mechanisms by which mycolactone may suppress local and systemic immune reactivity. In human monocytes expression of tumor necrosis factor alpha (TNF-α), interleukin-2 (IL-2), IL-10 and TNF-α induced NF-κB activation is suppressed by mycolactone *in vitro* in a dose dependent manner. Murine bone marrow derived macrophages produce low levels of TNF-α when infected with highly virulent but not with intermediately virulent or nonvirulent strains of *M. ulcerans* [64,66]. This finding is supported by another study where reduced TNF-α mRNA levels were found in activated macrophages 24h post infection [62]. At non-toxic concentrations, mycolactone inhibits functional and phenotypic maturation of human dendritic cells [63]. Their ability to activate allogeneic T cell priming and to produce inflammatory molecules is highly reduced. Interestingly, in these cells TNF-α production is only marginally affected [63]. Suppression of the production of chemokines by dendritic cells (DCs) may contribute to the observed lack of local inflammatory responses in BU lesions. Furthermore, it is likely that massive production of mycolactone in established lesions with large clusters of mycobacteria destroys infiltrating leukocytes, explaining why intact inflammatory cells are usually only found at the periphery of the necrotic areas [67].

Activities of mycolactone in animal models

When injected into guinea pigs, purified mycolactone produces the typical clinico-histopathological picture of BU with strong apoptotic and immunosuppressive actions [68]. Infection with mycolactone-negative *M. ulcerans* fails to induce ulceration in this animal model, but chemical complementation with purified mycolactone restores wild type pathology [61]. Histopathological analysis of guinea pig skin infected with wild type *M. ulcerans* revealed coagulative necrosis to appear already 24h post infection. Normal host cells were absent apart from small numbers of shrunken, pyknotic cells indicative for apoptosis. Infiltration of mononuclear leukocytes was present at the edges of the necrotic area 10 days post infection, some containing intracellular bacilli although advanced apoptotic changes were observed in these cells. Inflammatory infiltrates increased throughout a six week infection period but were always located at some distance from the bacterial foci [61]. In a mouse footpad infection model, significant differences in the mean footpad swelling - a macroscopic parameter for monitoring pathology - has been observed between a highly virulent strain from Benin, a moderately virulent strain from China and a mycolactone-negative mutant. Leukocyte infiltration two days post infection consisted predominantly of neutrophils. While the two virulent strains solely triggered an acute inflammation in the periphery of the necrotic area, mice infected with the mycolactone-negative mutant switched to chronic inflammation one week post infection with granuloma-like structures forming two weeks post infection and neutrophilic infiltrates vanishing concurrently [67].

A recently published work from Demangel et al shows that mycolactone diffuses into the tissue not only locally but also systemically through migrating monocytes [69]. In the mouse model structurally preserved mycolactone was detected in peripheral blood mononuclear cells (PBMCs), mononuclear cells of the lymph nodes (both draining and distant) and the spleen but not in the sera six weeks post infection. These findings support the hypothesis that the toxin exerts immunosuppressive effects at the systemic level.

Histopathological features of untreated BU lesions

Histopathological hallmarks of BU include the presence of extracellular clusters of AFB, massive contiguous coagulative necrosis, adipose cell ghosts and a remarkably low cellular infiltration [70,71]. In very early stages extensive involvement of subcutaneous fat in the form of a septate panniculitis and otherwise little inflammatory response apart from a slight histiocytic infiltration in the intermediate

area, between necrosis and healthy tissue has been described [72]. Bacteria are located focally and total numbers may vary greatly between individual lesions [73]. Currently BU lesions are not, like leprosy lesions, routinely classified into paucibacillary and multibacillary forms, but a deeper understanding of the influence of host factors on *M. ulcerans* disease progression may lead in future to such a classification system. In contrast to *M. tuberculosis* and *M. leprae*, *M. ulcerans* has traditionally been classified as an extracellular pathogen [74]. However, in 2002, Drancourt et al were able to grow *M. ulcerans* inside amphibian cells *in vitro*, and three years later Coutanceau et al described intracellular bacteria in a mouse model of BU [62]. Recently, Torrado et al provided evidence for a transient intra-macrophage growth phase in the mouse model and subsequent release into the surrounding. In specimens from BU patients this phenomenon is usually not explicitly appreciated, as bacilli and inflammatory exudates are not always concomitantly represented. An additional reason may be that first clinical symptoms leading to the collection of a diagnostic biopsy or surgical excision of the lesion may occur substantially later than an early phase of the infection, in which intracellular replication may potentially play a major role.

Local and systemic immune responses in BU patients

Humoral immune responses

Currently only limited data on the development of *M. ulcerans* specific immune responses are available and it is not entirely clear to what extent mycolactone secretion may lead to a suppression of systemic immune responses. The control of *M. ulcerans* infection may be primarily dependent on cell-mediated immunity involving activated macrophages, T cells, and Th type 1 cytokines, as is thought to be the case for *M. tuberculosis* and *M. leprae* infection. However, also antibodies could provide 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.

In tuberculosis (Tb) endemic areas, where BCG vaccination is common, broad antigenic cross-reactivities of *M. ulcerans* antigens with *M. tuberculosis* and *M. bovis*

antigens complicate the analysis of *M. ulcerans* specific immune responses substantially. In studies with *M. ulcerans* culture filtrates IgG antibody responses against the secreted *M. ulcerans* proteins were frequently found in BU patients, but also in Tb patients from BU non-endemic regions [75] [76]. IgM responses of BU patients against the filtrate proteins were more distinct than those of healthy family members living in the same village [76], indicative for B cell stimulation. Diaz et al. used the highly immunogenic *M. ulcerans* 18 kD small heat shock protein, which has no homologues in *M. bovis* and *M. tuberculosis* to monitor *M. ulcerans* specific IgG responses in BU 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 [77]. This is indicative for the development of specific humoral responses against *M. ulcerans* in exposed, but otherwise healthy individuals. Infection with *M. ulcerans* may thus lead only in a minority of exposed individuals to clinical manifestations. Most of the others may develop transient infection foci and even nodular lesions may resolve spontaneously [78]. Immune responses in healthy household contacts have also been described in an Australian study [79], where a lower background staining than with African sera facilitated analysis with *M. ulcerans* cell extracts.

Systemic cellular immune responses

While patients with early BU lesions rarely show delayed-type hypersensitivity responses to intradermally injected *M. ulcerans* lysate, reactivity is common in patients with healing lesions [75,80]. However, it is currently not clear to what extent BU patients can mount a *M. ulcerans* specific Th1 response. Triggering of pattern recognition receptors by structural components of *M. ulcerans* could lead to phagocyte activation and production of proinflammatory cytokines, including TNF-α, IL-1ß, and IL-12, and also anti-inflammatory cytokines, including IL-10 and transforming growth factor beta. IL-12 could in turn drive T cells and NK cells to produce Th1 proinflammatory cytokines, including IFN-γ and TNF-α. In synergy with TNF-α, IFN- γ could activate *M. ulcerans* infected macrophages, leading to elimination rather than intracellular multiplication. On the other hand, IL-10, an anti-inflammatory cytokine produced by differently activated macrophages, Th2 and subsets of regulatory T cells and DCs could down-regulate IL-12 production leading

to a decrease in IFN-γ production. Several research groups, using different experimental approaches, have analyzed expression profiles of these key cytokines and proliferative responses of peripheral blood mononuclear cells (PBMCs) of BU patients stimulated *ex vivo* with mycobacterial antigens and immunostimulatory compounds (Table 1). Wide individual variations in IFN-γ and IL-10 responses have been found in these studies, which may be one reason accounting for discrepancies with respect to the influence of the disease stage on the polarization of the Th response [81,82,83,84]. In a case report acquired Th cell anergy following infection with *M. ulcerans* has been described in a BU patient from Australia [85]. Before infection, the patient's PBMCs responded to *ex vivo* stimulation with *M. ulcerans* by producing Th1 cytokines, but, after development of BU, a shift toward production of Th2 cytokines was found. Taken together the various studies are indicative for a generalized impairment in IFN-γ production during active BU. The capacity to produce IFN-γ is also depressed in Tb [86,87,88,89], where cellular components of *M. tuberculosis* have been shown to down-regulate IFN-γ receptor signaling [90,91]. Similarly, in BU mycolactone may also not be the only component with immunosuppressive activity. Data from Yeboah-Manu et al have shown that PBMCs recover from the systemic downregulation of IFN- γ production in BU after surgical removal of the lesion [92] containing the vast majority of mycobacterial material. It should be noted that many conditions frequently found in rural Africa, including infections such as malaria [93], helminthiasis [94,95,96] and Tb [88], but also malnutrition [97] can lead to a partial suppression of cellular immune responses as well.

Local immune response in untreated lesions

In BU lesions inflammation is usually minor with respect to the extent of necrosis of subcutaneous tissues and dermal collagen [72,74]. There may be a constant influx of neutrophils, monocytes/macrophages, and lymphocytes to the active lesions, but mycolactone produced by clusters of mycobacteria is likely to induce apoptosis and necrosis of the inflammatory infiltrate [67]. In particular remainders of early neutrophilic infiltrates may still be immunohistochemically detectable inside the necrotic areas [98].

After long periods of persistent infection, granulomatous reactions with epithelioid macrophages, variable numbers of giant cells of the Langhans type, peripherally situated lymphocytes, and relatively few AFB develop [72]. Granuloma formation is more often found in ulcerative lesions than in pre-ulcerative lesions [74]. In particular ulcer edges may show signs of chronic inflammation accompanied by a mixed pro-inflammatory/anti-inflammatory cytokine balance [99,100]. Vast apoptotic/necrotic regions with minimal inflammation may thus be surrounded by a rim of mixed leukocyte infiltration. Healing is associated with the development of granulation tissue, fibrosis, and granulomatous inflammation [70]. New *M. ulcerans* satellite colonies may still develop in the lesion after the onset of granulomatous inflammation [73]. Analyses of intralesional cytokine production patterns have yielded conflicting results (Table 1) with respect to the polarization of the local response [81] [101] [83,100]. Within a lesion the distribution of different types of leucocytes is extremely heterogeneous [73]. Therefore results based on the analysis of single biopsies may not be representative for the entire lesion [102]. In addition, it appears that there is a great variation in individual response patterns to *M. ulcerans* infection. A gradation system used for the classification of inflammatory responses in leprosy, ranging from foamy macrophages (lepromatous) to well-formed granulomas (tuberculoid), may also be useful for the classification of BU lesions.

Local immune responses during chemotherapy

In active BU disease, a protective cloud of mycolactone around the mycobacterial clusters is thought to both destroy infiltrating leukocytes and hinder them from passing pro-inflammatory signals to other cells [63]. It is most likely, but still remains to be proven experimentally, that mycolactone production is reduced or abolished early after the onset of rifampicin/streptomycin chemotherapy due to bacterial growth arrest and/or cell death. Declining toxin levels allow leukocytes to reach the extracellular mycobacteria, leading to their phagocytosis and destruction [98]. Chronic leukocyte infiltration cumulates in the development of ectopic lymphoid structures [98].

After eight weeks of chemotherapy antigen presenting cells as well as B and T lymphocyte foci are found in large numbers inside the BU lesions [98]. Gathering of DCs around the necrotic core as well as development of defined granuloma structures indicates that antigen recognition and processing is leading to active *M. ulcerans* specific immune responses [98]. Three major types of mixed infiltrates, differing in cellular composition, architecture and localization have been identified in the treated lesions: (1) highly organized epithelioid granulomas primarily located in deeper dermal tissue; (2) less organized diffuse infiltrates in all areas of the dermal connective and adipose tissue; (3) dense lymphocyte clusters in proximity to vessels. The outer layer of granulomas is mainly composed of T lymphocytes with $CD4^+$ T cells outnumbering $CD8^+$ T cells. These lymphocyte belts are interspersed with dermal DCs. Focal clusters of B cells appear at the outer margins of the T lymphocyte layer. Antigen-presenting cells, in particular Langhans' giant cells and epithelioid macrophages form the centre of granulomas. Many lymphocytes are positive for activation and proliferation markers. Neither PMNs nor NK cells contribute significantly to granuloma formations. While in human Tb granulomas develop a central necrotic core, this is not observed in the lesions of the antibiotic treated BU patients. Here, the centre is mainly formed of foamy histiocytes and Langhans' giant cells, like reported for leprosy. Mycobacterial material is only infrequently detected inside these structures. BU granulomas thus may function primarily as a place for antigen presentation and adaptive immune response rather than for sequestration of the mycobacteria.

In contrast to granulomas, regions of diffuse cellular infiltration in the dermal connective tissue contain sparsely distributed PMNs with sporadic focal clusters near necrotic areas. Small clusters of B lymphocytes are found, but T lymphocytes and foamy histiocytes are the most prominent cell types. Also here a large proportion of lymphocytes stain positive for activation proliferation markers. Macrophages/monocytes are particularly abundant in adipose tissue and around necrotic areas. The frequency of epidermal Langerhans cells is strongly elevated and dDC are distributed throughout diffuse mixed infiltrates with increasing density towards the margins of necrotic areas. Appendices of dDC reach into the damaged tissue. Vast amounts of shed CD14 is observed, which is implicated in the clearance of necrotic tissue [103]. Adjacent to lymphatics or blood-vessels follicle-like

structures with dense aggregations of lymphocytes are found. Here B lymphocytes are the dominating cell type followed by T lymphocytes with $CD4^+$ T cells being more frequent than $CD8^+$ T cells.

After eight weeks of chemotherapy, AFBs with beaded appearance may still be found inside large necrotic areas. In other areas rod-shaped AFB have largely disappeared. However, acid-fast mycobacterial debris that can still be stained with antibodies specific for mycobacterial antigens, is still abundant and primarily located inside mononuclear phagocytes. AFB start to be internalized by phagocytes already after two weeks of treatment, when *M. ulcerans* can still be cultured from tissue homogenates (unpublished results). Also cellular infiltrates and small B cell clusters are already observed at this time point. Antibiotic treatment may thus act rapidly on the bacterial metabolism and interfere with mycolactone production, while complete killing of bacteria takes at least four weeks of chemotherapy. After four weeks of antibiotic therapy granulomas have not yet been fully developed and Langhans' giant cells are still rare (Schütte et al, in press), but a shift of infiltrating cells from acute to chronic subsets may already have taken place. Abundant clusters of AFB inside necrotic tissue may still be found (Figure 3), surrounded by dense bands of acute and chronic infiltrates (Schütte et al, in press). Abscess formation by PMNs is occasionally found, which may lead to secondary tissue damage and "reactivation" phenomena such as ulceration or painful papules weeks to months after completion of treatment (Schütte et al, manuscript in preparation).

Marked heterogeneity in response patterns of individual BU patients may be observed with respect to both clinical outcome and histopathological findings. This may be related to factors such as: (1) lesion stage and size and extent of necrosis (2) total burden of *M. ulcerans* inside a lesion and (3) composition and organizational status of cellular infiltrates. It is not clear, which factors play a crucial role in the development of ectopic lymphoid tissue during chemotherapy of BU. Plasmacytoid and myeloid DCs present in BU lesions prior to antibiotic treatment [102] and lymphoid tissue-inducer cells may play an important role. Although tertiary lymphoid tissue is thought to develop in infectious diseases primarily to sequester pathogens, this process is often accompanied by tissue damage. Occasionally antibiotic treatment thus may even accelerate the progression of early plaques and oedemas

to ulcerative lesions. In some patients new ulcerations develop in the course of or after completion of antibiotic treatment. These are usually not associated with the development of new infection foci (Schütte et al., unpublished results), but are most likely located at sites containing preexisting unrecognized infection foci in which mycobacterial antigens and immunostimulators lead to massive local immune responses .

Conclusion

The pathogenesis of BU is strongly associated with the cytocidal and immunosuppressive activities of the macrolide toxin mycolactone. Histopathological hallmark of progressing disease is a poor inflammatory response despite of clusters of extracellular bacilli. While traditionally wide excision of the infected tissue was the standard treatment, provisional WHO guidelines now recommend eight week chemotherapy with streptomycin and rifampicin. Soon after start of the antibiotic-therapy onset of vigorous local immune responses is observed, which speaks for a rapid decline of mycolactone levels in the lesion. Three different general types of infiltration, diffuse mixed infiltrates, granulomas and dense lymphocyte aggregation in the vicinity of vessels are found in the treated lesions. This structural differentiation may reflect a range of different functional activities required for resorption of necrotic tissue and complete clearance of residual mycobacterial cells and antigens. After eight weeks of antibiotic treatment necrotic regions are surrounded by large numbers of dDCs and their appendices reach into the damaged tissue, indicating enhanced antigen uptake and presentation. A large proportion of lymphocytes in the infiltrates are expressing activation and proliferation markers. It is likely that the curative effect of the antibiotic treatment is supported by these immunological reactions. Future studies on re-infection rates will show, whether chemotherapy of BU results in long-lasting immunity and protection against re-infection by *M. ulcerans*.

Expert opinion

There is convincing evidence that the macrolide toxin mycolactone of *M. ulcerans* plays a crucial role in the pathogenesis of the chronic necrotizing skin disease BU. Tissue destruction, killing of leukocytes that are attracted to the site of a *M. ulcerans* infection and possibly also some downregulation of systemic immune responses can be primarily attributed to the apoptotic and direct bactericidal activities of the toxin. However, the exact molecular mode of action of mycolactone is not known, nor has its distribution and stability in the host been studied. The fact that tissue destruction reaches far beyond the tissue areas containing large clusters of extracellular *M. ulcerans* demonstrates that mycolactone diffuses into the tissue. Development of leukocyte infiltrates in BU lesions already after one to two weeks of chemotherapy indicates on the other hand that the stability of mycolactone in the necrotic tissue is limited. However, this represents only indirect evidence for the behavior of mycolactone in BU lesions. Due to lack of a sensitive and robust quantification method for mycolactone in biological samples it is currently also not clear, whether mycolactone related metabolites could potentially be detected in the blood or urine of Buruli ulcer patients – a feature that could be used for the development of a specific diagnostic test. Various attempts are therefore currently made to develop an immunological antigen capture assay or a mass spectrometry-based test system for mycolactone. The macrolide toxin could also represent a suitable target for the development of a toxoid-based vaccine. However all approaches to develop a protein conjugate that elicits high titers of mycolactone-specific and potentially neutralizing antibodies have failed so far and only one mycolactone specific monoclonal antibody has become available (unpublished results). There is also no evidence for the development of natural immune responses against mycolactone in BU patients or *M. ulcerans* exposed, but healthy individuals.

In established *M. ulcerans* disease the vast majority of AFB are located extracellularly in necrotic areas of the lesions. However results from experimental infections in mice speak for a transient intracellular stage early in the infection. The bacteria appear to kill the phagocytes that have ingested them after a few days and may then develop further into microcolonies, which develop a protective cloud of mycolactone around them. Since human biopsies or excised tissue is only becoming available at an advanced stage of the infection it is not entirely clear, whether the same holds true for the infection in humans. However, at the periphery of necrotic

areas in established human BU lesions, encounter of spreading *M. ulcerans* cells with human phagocytes can be observed. Here small numbers of intracellular AFB are found. Signs of apoptosis at the side of the leukocytes and 'beaded' appearance of the mycobacteria upon ZN staining indicates that this encounter can be lethal both for the invader and the host cells. New satellite colonies and lesions may develop from spreading mycobacteria, but this seems to be a relatively rare event. Probably only a few spreading mycobacteria succeed in developing satellite cluster of mycobacteria that are large enough to form a protective cloud of mycolactone. Host factors may largely determine whether a patient develops a single or multiple skin lesions or even metastatic osteomyelitis. The most striking finding of immunological analyses in BU patients is the wide individual variation in cytokine responses both with respect to magnitude and response patterns. This may make it impossible to associate different disease stages with either a Th1 or a Th2 dominated response. However, a more differentiated gradation system for inflammatory responses in BU may turn out to be as useful as the differentiation between lepromatous and tuberculoid leprosy. Like in leprosy, also in BU paucibacillary and multibacillary forms seem to exist, but this aspect has also not been systematically investigated.

Only a subgroup of individuals exposed to *M. ulcerans* appears to develop clinical disease. This indicates that the human immune system is in principal able to cope with a *M.* ulcerans infection. Spontaneous healing of lesions may occur after prolonged periods of progressive ulceration, but also nodular stages may occasionally resolve spontaneously. Granuloma formation and conversion to positive burulin skin test has been associated with spontaneous healing, suggesting a role for cellular immune mechanisms. But also the humoral arm of the adaptive immune system may contribute to host defense against the largely extracellular *M.* ulcerans. BU granulomas do not have a necrotic core containing intact mycobacteria, as is found in tuberculosis and there is also no other histopathological evidence for the development of latent stages of *M. ulcerans*. However, relapses observed after prolonged disease-free intervals in some patients may not only be caused by re-infection.

Current treatment options for BU include surgical excision, chemotherapy and heat treatment. While chemotherapy has for a long time been regarded as largely

ineffective for *M. ulcerans* infection, treatment with a combination of rifampicin and streptomycin is currently being re-evaluated with encouraging results. While surgical excision aims to remove tissue containing bacterial clusters completely, tissue and bacterial cell debris remains after antibiotic therapy, in particular in the case of pre-ulcerative lesions, in the body. Mycobacterial antigens and immunostimulators can in turn attract strong leukocyte infiltration and the formation of abscesses. Development of ectopic lymphoid tissue in the lesions demonstrates that BU patients are capable of raising local anti-mycobacterial immune responses once the mycolactone levels decline. These inflammatory reactions may slow down wound healing after successful chemotherapy. Interestingly, thermotherapy is associated with much less vigorous local cellular immune responses (unpublished results). Massive immunostimulation seems to lead in some patients to apparent reactivations of the disease weeks after completion of antibiotic therapy. Secondary ulcerations may be observed, which are not associated with the development of new mycobacterial foci (unpublished results).

Differentiated treatment strategies for different categories of BU lesions, combining the available treatment options, need to be developed and refined. With an improved understanding of BU pathogenesis and protective immune responses it may also be possible to develop both simple point of care diagnostic tests and a vaccine for targeted protection of the populations living in BU endemic areas.

Figures

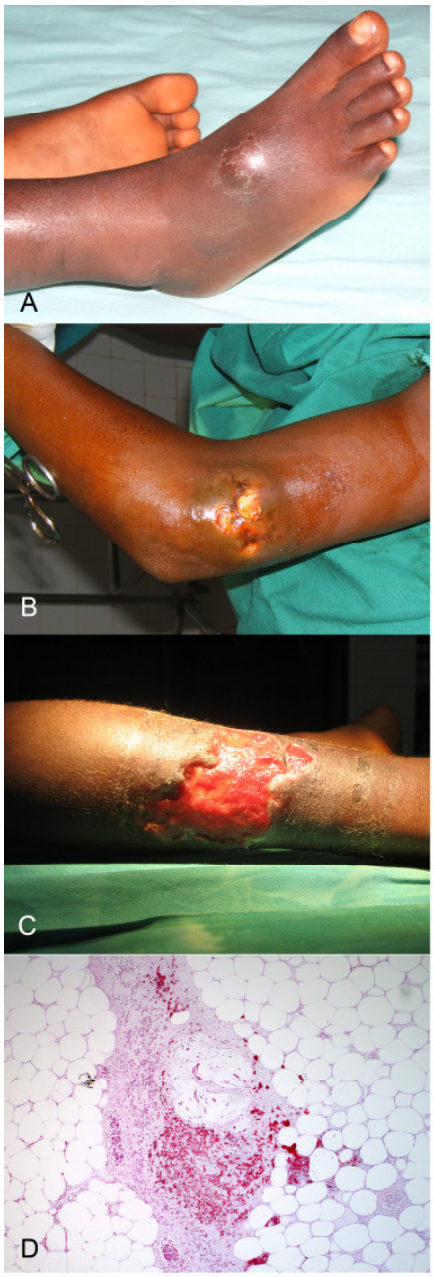

Figure 1. *Clinical stages and histopathology of M. ulcerans infection*
(a) Nodular, (b) early ulcerative and (c) late chronic disease stage. (d) Typical histopathological picture of BU with masses of acid-fast rods lying in the necrotic subcutaneous tissue (Ziehl Neelsen staining on paraffin section, original magnification 10x).

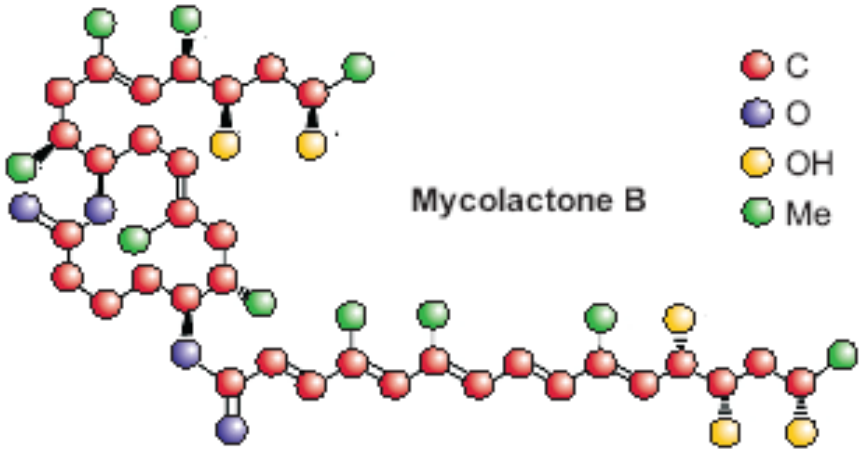

Figure 2. Structure of Mycolactone B

Author	Year	Country	Study population	Lesion type	Immune response	Sample	Stimulation	Analysis	IFN-γ	IL-10	Others
Gooding et al.	2001	Australia	Patients/ healed	Ulcers	Systemic	PBMC	Living *M. ulcerans*/ living BCG	FACS/ ELISA	Absent in both acute or healed disease	-	Very low to absent proliferation of PBMCs
Gooding et al.	2002	Australia	Healed/ household contact	Ulcers	Systemic	PBMC	Living *M. ulcerans*/ living BCG	mRNA/ ELISA	Present in contacts, absent in former patients (both mRNA and protein)	Absent in contacts, present in former patients	IL-12 in contacts, IL-4, -5, -6 in former patients
Prevot et al.	2004	French Guyana	Patients	Nodules/ ulcers	Local	5mm biopsy	-	mRNA	High transcripts in nodules, low in ulcers	Low transcripts in nodules, high in ulcers	Slightly lower TNF-α transcripts in ulcers
Prevot et al.	2004	French Guyana	Patients	Nodules/ ulcers	Systemic	PBMC	Whole killed *M. ulcerans* and BCG	ELISA	Lower in patient vs. healthy controls/ lower in ulcers vs. nodules	Higher in patients vs. controls/ higher in ulcers vs. nodules	BCG stimulation relatively ineffective
Westenbrink et al.	2005	Ghana	Patients	Early stage (active BU or surgery <42 days ago)/ late stage (surgery >42days ago or healing)	Systemic	Whole blood	PHA, PPD	ELISA	Higher in late stage than early stage disease	No difference between groups	-
Yeboah-Manu et al.	2006	Ghana	Patients before and after surgery (5 months)/ household contacts	Nodule, plaque, ulcer	Systemic	PBMC	IPP, PPD, IRIV, PHA	ELISpot/ ELISA	Increasing presence after surgical removal of lesions	n.d.	No variation in IL-12 production
Phillips et al.	2006	Ghana	Patients/ healed BU/ household contacts	Nodules/ ulcers	Systemic	Whole blood	*M. ulcerans* or *M. tuberculosis* sonicate	ELISA	Slight increase in ulcer vs. nodule, highest in healed/ no difference between patients and contacts	Highest in ulcers/ low in contacts	No differences when stimulated with Tb sonicate
Kiszewski et al.	2006	Benin	Patients	Ulcers (with or without granulomas)	Local	Tissue section	-	IHC	Strong increase in sections with granulomas vs. without	Slight decrease in granulomas	Increase of CD8 with granulomas/ no change in TNF-α and TGF-β
Phillips et al.	2006	Ghana	Patients	Nodules/ ulcers	Local	Punch biopsy	-	mRNA (relative to 18S)	Marginal differences (not significant)	Marginal differences (not significant)	Much higher IL-1β and IL-8 in ulcers than nodules

Peduzzi et al.	2007	Ghana	Patients	Nodules/ ulcers	Local	Whole surgical excision	-	mRNA (relative to β-actin)	Very low to absent	Focal peak in one patient	Great variation of TNF-α levels throughout the lesions
Schipper et al.	2007	Ghana	Patients/ healed BU	Pre-ulcerative/ ulcerative (necrotic, organizing, granulomatous)	Local	6mm punch biopsy	-	IHC	Rising from necrotic over organizational to granulomatous stage of infection	Strong in endothelium, lower in epithelioid and giant cells	Strong TNF-α staining
Schipper et al.	2007	Ghana	Patients/ healed BU	Pre-ulcerative/ ulcerative (necrotic, organizing, granulomatous)	Systemic	Whole blood	PHA, PPD	ELISA	Higher in patients with ulcerative and healed than pre-ulcerative lesions and in controls	Higher in patients with pre-ulcerative and healed than with ulcerative lesion or in controls	No differences with or without BCG scar

Table 1. In vivo data on local and systemic cytokine responses in BU patients

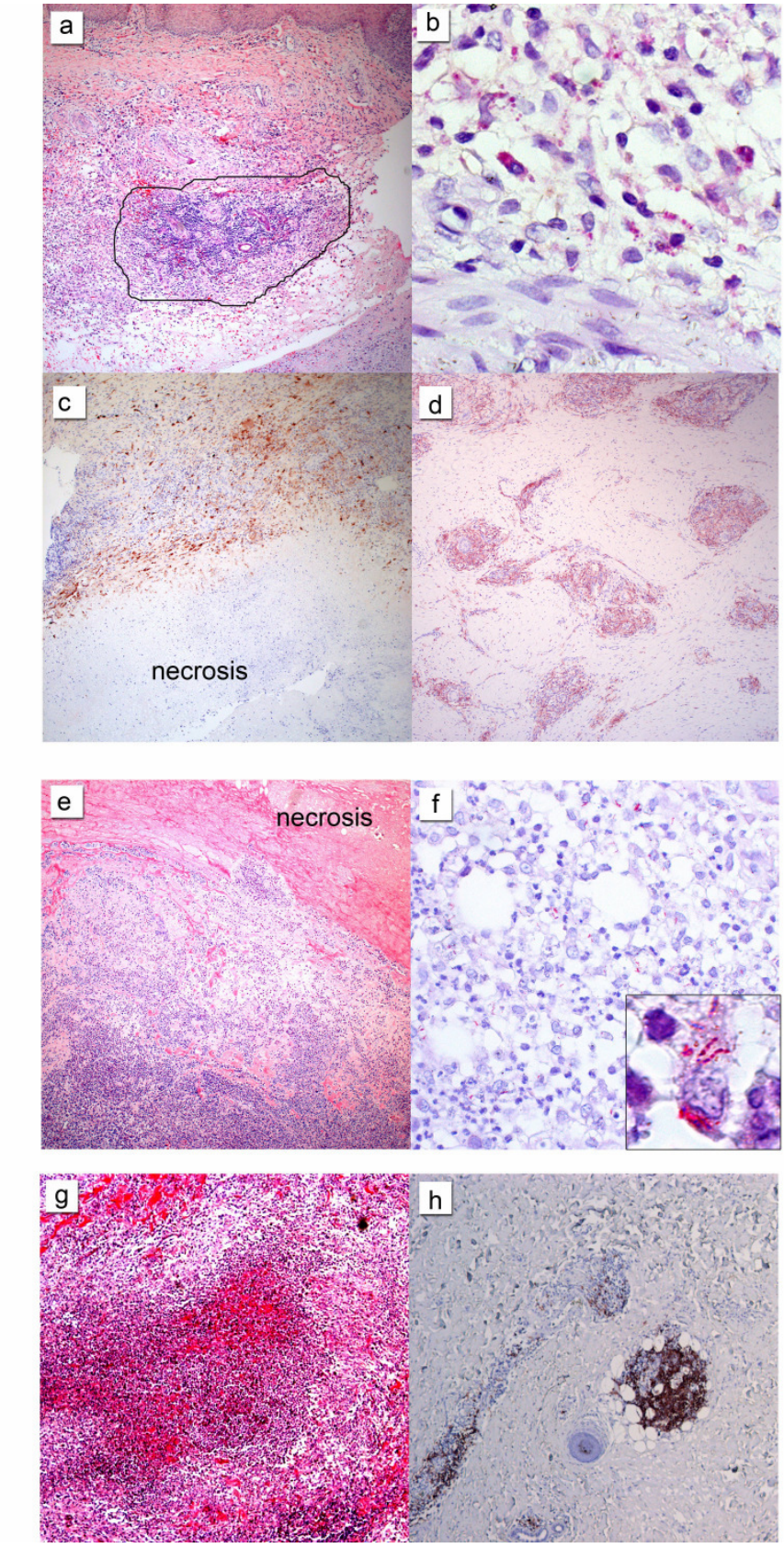
a
b
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Figure 3. *Histopathological changes during chemotherapy*

(a-d) Ulcerative lesions after eight weeks R-S treatment. (a) Follicle-like lymphocyte aggregation. (b) Acid-fast mycobacterial debris inside macrophages. (c) Large numbers of S100 positive DCs surrounding a necrotic lesion core. (d) CD14 positive granulomas in the macroscopically healthy lesion margin. (e) Ulcer after four weeks of R-S treatment. Dense mixed leukocyte infiltration at the necrotic border. (f) Early nodule after four weeks of R-S treatment. Intracellular AFB phagocytosed by macrophages. AFB show signs of degradation (“beading”). (g) Plaque after twelve weeks of R-S treatment. Abscess formation by PMNs. (h) Plaque after two weeks of R-S treatment. Distinct CD20 positive B lymphocyte cluster.

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CHAPTER 12

General Discussion and Conclusions

12.1. General remarks

In 1998 WHO established the Global Buruli Ulcer Initiative to coordinate BU control and research efforts. Although research on BU has been intensified since then there are still many open questions regarding reservoir, transmission, pathology, immunology and epidemiology. Furthermore, improvements are necessary with respect to health care, prevention, surveillance and awareness of the population. To efficiently fight BU and bring this neglected tropical disease out of obscurity, endemic countries, NGOs and research institutions actively collaborate and mobilize expertise and resources worldwide.

Improved case detection and awareness for the disease of populations in endemic areas has led to a better estimation of overall incidence rates. Over 30 countries worldwide are affected [1] and more BU foci are likely to be discovered in future, especially in sub-Saharan Africa. The most endemic countries such as Benin, Ghana and Côte d'Ivoire report already 1,000 to 2,000 new cases per year [2]. Assessing the burden of BU and establishing a control programme in endemic countries with limited resources is very demanding as health care and research infrastructures in those countries are usually poorly developed.

Well trained clinicians and health workers who are capable of diagnosing and treating the disease are rare, and although community participation in the recognition of disease symptoms is advancing, patients often present late with severe ulcerative lesions. In many BU endemic regions of Africa sufficient resources to treat and rehabilitate these patients are not available. However, more support is gradually becoming available to promote research on better diagnostic, treatment and preventive tools and to foster cooperation among countries in order to strengthen surveillance, control and rehabilitation.

The discussion section of this work is reviewing the current diagnosis and treatment strategies for BU as well as the prospects for vaccine design.

12.2. Diagnosis of BU

In an area known to be endemic an experienced person can diagnose BU on clinical grounds [3], although other ulcerating or boil forming diseases can be confused with *M. ulcerans* infection. Current goal of WHO is to reconfirm at least 50% of the clinically diagnosed BU patients by laboratory analyses. With the introduction of antibiotic therapy of BU in 2004 [4], the necessity of laboratory confirmation became even more crucial. Four laboratory-confirmatory methods are used retrospectively on samples taken during treatment, namely AFB examination of smears and biopsies, culture, PCR and histopathology [5].

12.2.1. AFB, culture and PCR

Exudate smears can only be obtained by taking swabs from ulcerative lesions and microscopic examination for AFB is associated with low sensitivity. However, it is the only yet available field test which can be carried out rapidly at local facilities. Culture of *M. ulcerans* is an extremely time-consuming technique [6], that requires skilled personnel. It yields false-negative results too often to be implemented for routine diagnostic purposes, but has recently been used to monitor the kinetics of bacterial killing under antibiotic therapy [7]. PCR is a test with a sensitivity of up to 100%, which often allows confirmation of clinical diagnosis even when samples are culture and smear negative. Although the target sequence IS2404 has also been found in some other environmental bacteria [8], positivity of clinical specimens is a strong indication for a *M. ulcerans* infection. Swabs or biopsies can both be assessed within two days, but in West Africa the expensive equipment and demanding technical skills, inevitable to perform this method, are only available in a few reference laboratories. Furthermore, the high risk for false positive results caused by contamination requires strict quality control, which is not yet implemented. Therefore, the introduction of inter-laboratory comparable protocols has been suggested and will be established in the near future. We have used real-time PCR, which is less prone to yield false positive results than conventional PCR, for diagnosis in our thermotherapy study. In combination with histopathology and AFB staining it allowed to unambiguously reconfirm clinical diagnosis (see Chapters 7 and 8).

12.2.2. Histopathology

BU lesions develop a distinctive pathology at the site of infection which allows an experienced pathologist to diagnose the disease on these grounds [9]. Sampling has to be performed with great care to obtain tissue from the presumed lesion core or from ulcer edges. Biopsies must include all levels of the integument as focal bacterial clusters and the associated pathological changes are primarily located inside the adipose region of the deeper dermal layer [10]. Though histopathology is a reliable diagnostic tool, it requires special equipment and versed scientists to be performed and takes at least four days for sample processing and evaluation. Recently, punch biopsies have been introduced as standard procedure in the diagnosis of BU allowing histological analysis of tissue from both ulcerated and early closed lesions [11]. Due to the heterogeneity of lesions and the superficial nature of biopsies, sensitivity is much lower than for major excision samples.

Given proper processing and storage, tissue embedded in paraffin can be preserved over years and used not only for diagnosis, but also additional research, as it is shown in the present work (see Chapter 6). Histological analyses can provide insight into processes taking place during progressing disease [12], treatment [13] or healing [14] and are extremely valuable to better understand the natural history of the disease (see Chapters 3, 4 and 5). The complete “lack of local immune responses” was thought to be a hallmark of progressing BU disease and it was generally assumed, that the onset of a granulomatous response marks the beginning of containment and eventual healing of a *M. ulcerans* infection [15]. More detailed histological analysis is now modifying this point of view. Ulcer edges show signs of chronic inflammation accompanied by a mixed pro-inflammatory/anti-inflammatory cytokine balance as consistent histopathological features [16]. It was demonstrated that new satellite lesions can still develop after the onset of these cellular immune response followed by granuloma formation [17]. It appears that infection with *M. ulcerans* does trigger a local immune reaction, but that infiltrating leukocytes are subsequently destroyed in areas with high mycolactone concentration. This may explain the observation of vast apoptotic/necrotic regions surrounded by a rim of mixed leukocyte infiltration. In our immunohistological studies remainders of early neutrophilic infiltrates were still detectable inside the necrotic areas. In chronic progressing ulcerative lesions

granulomas are commonly observed at the lesion edges [16,17]. This may represent an attempt of the immune system to fend off and isolate the invading mycobacteria.

A second conclusion, that *M. ulcerans* is an extracellular pathogen unlike other mycobacteria, was first challenged in 2005, when a transient intraphagocytic stage was observed in the animal model [18,19]. Examination of the peripheries of necrotic acellular areas in BU patients revealed that inflammatory infiltrates containing bacilli are consistently present during infection [16,20]. This phenomenon is not often appreciated because inflammatory exudates and bacilli are not always represented in the tissue fragments available for analysis. Monitoring the early development of experimental *M. ulcerans* infection of mice indicates a great proportion of bacilli replicating inside phagocytes before their final release into the tissue due to mycolactone cytotoxicity (Ruf et al, unpublished data). In future, histological analysis of very early BU lesions should be conducted to better understand the factors contributing to the establishment of *M. ulcerans* infection such as intra- and extracellular bacterial stages and kinetics of local immune responses. There is mounting evidence that a proportion of *M. ulcerans* infections resolve spontaneously before giving rise to clinical disease.

12.2.3. Future improvements

Novel strategies to develop highly predictive but simple “point-of-care” diagnostic tests allowing fast confirmation of BU at peripheral treatment centres are considered a high research priority. Serological studies have indicated that exposure to *M. ulcerans* leads only in a minority of individuals to clinical symptoms [21]. One focus on the way to a field-compatible diagnostic test could be the development of an antigen capture assay based on antibodies against unique immunodominant proteins of *M. ulcerans* such as its 18kD small heat shock protein (shsp) [21]. Immunohistochemistry (IHC) performed with monoclonal antibodies (mAbs) against this surface-bound protein allows specific and sensitive detection of the pathogen in tissue and culture (see Chapter 10). The final goal is a cheap and easy to handle test format such as a dip-stick, requiring only small amounts of urine or blood.

Another focus is on mycolactone, the macrolide exotoxin, which is supposedly more widely distributed inside the lesion than the bacilli. Antibodies against this small molecule are not easily elicited and only one mycolactone-specific mAb has been generated so far (Dangy et al, unpublished data). A phage-display based approach is currently conducted as alternative strategy for the generation of mAbs with alternative specificity. The available mAb against the macrolide is currently under investigation for potential use in histopathological analysis of frozen sections (unpublished data). Since mycolactone is ethanol-soluble, paraffin-embedded thin sections are not suitable for such analyses. Excised tissue could be assessed for mycolactone distribution not only for diagnostic purpose, but also to obtain more detailed information about extent and severity of infection. In addition, decline of mycolactone levels could represent a good surrogate marker for the efficacy of antibiotic or heat treatment.

12.3. Treatment of BU

12.3.1. Antibiotics

Historically, surgery is the front-line therapy for all stages of BU [22,23]. However, only a minority of BU patients in West Africa had access to surgical treatment centres. The introduction of an efficacious antibiotic combination is currently shifting the balance from mere surgery to antibiotic therapy with or without surgical intervention [4]. In African countries daily administration of both rifampicin and streptomycin is now used at a number of treatment centres after a clinical trial and observational studies showed its efficacy in humans [7,24]. Large observational studies indicate that 30-50% of patients can be cured with antibiotics alone, primarily those with early pre-ulcerative or small ulcerative lesions. Patients who recover without additional surgery most often suffer from a minor lesion less than 5cm in diameter. Between experts, the rifampicin-streptomycin regimen remains a subject of debate and, although promising, has to be improved in several ways [25]. Streptomycin is contraindicated during pregnancy and although it has been reported to be well-tolerated, there is a risk of renal and ototoxicity in particular in children [26]. Several studies to simplify antibiotic treatment are ongoing. In the mouse footpad model, five days per week R-S administration are sufficient to cure *M. ulcerans* infection [27] and the same concept

is currently under evaluation in a pilot dose-finding study in Ghanaian BU patients [28]. Trials to evaluate the potential of the orally administered antibiotic clarithromycin, which has excellent bio-availability and penetration into tissue and good anti-mycobacterial activity *in vitro* [29], are under way (van der Werf, drug trial NCT00321178). This macrolide penetrates phagocytes and accumulates in organelles delivering the drug directly to the site of infection [30]. Even a treatment course of six months can be followed with this combination and curing severe disseminated cases becomes imaginable [31].

12.3.2. Thermotherapy

The currently most promising alternative approach is BU therapy through local heat application. A small proof-of-principle trial used non-hazardous commercially available bags containing phase-change material to locally apply temperatures around 40°C over several weeks (see Chapters 7 and 8). Amazingly rapid re-attachment of undermined skin and subsequent healing processes were achieved. Results from this "proof of principle" trial strongly suggest that heat kills or suppresses *M. ulcerans* in the affected tissues and, at the same time, induces a local immune response which is carrying the healing process further. These promising results encouraged implementation of a larger dose-finding trial and the recruitment of complicated cases. Exploration of the turning point is important to determine the heat application schedule sufficient for the host's own defence and healing to take over. Furthermore, an "over-night" treatment schedule would be very convenient for both patients and health workers. After completion of the dose finding study field workers have to be trained to independently administer heat-therapy according to the appropriate schedule. This would allow the realization of a community effectiveness trial, where thermotherapy is transferred to the community to accomplish "on-the-spot" treatment of BU patients. The non-invasive heat application was already greatly appreciated by all participants during the pilot study. Broad acceptance of a treatment modality by both health workers and the local population is thus likely to be a major advantage. In regions with limited resources, where the disease is the most prevalent, thermotherapy may open up a new era in the treatment of *M. ulcerans* infections and could become a supplementary if not alternative treatment option in the fight against BU.

12.3.3. Mycobacterial viability studies

As noted already, histopathology is a good tool to evaluate local immune responses and bacterial distribution during therapeutical interventions. Nevertheless, efficacy evaluation of a particular treatment should also include determination of mycobacterial viability which is, apart from performing time-consuming and insensitive culture, not possible to date.

A possible marker of viability loss is the so-called “beading” of mycobacteria. Microscopy can be used to evaluate the effects of therapy on *M. leprae* as it is well known that even slight physical damage to the bacterial cell wall is sufficient to change the acid fast properties visualized by “beaded” ZN staining [32,33]. At close examination, the same phenomenon was observed in mouse footpad *M. ulcerans* lesions (Ruf et al, unpublished data) and human BU (see Chapter 6) treated with antibiotics for several weeks. Unfortunately, if there is sufficient residual cytoplasm to outline the bacillus, thereby appearing solid in ZN staining, the degenerated form can only be identified by electron microscopy [33]. Therefore, this method is too insensitive to monitor viability, but may be used to give a rough estimate of treatment success or probability of relapse.

Another surrogate marker to measure viability may be mycolactone content of clinical specimens. The observed recovery of local immune responses associated with declining necrosis speaks for a reduced mycolactone release during R-S treatment (see Chapters 3, 4 and 5). Lipid extracts of punch biopsies from BU patients with and without antibiotic treatment could be assessed for mycolactone levels via mass spectroscopy (Wansbrough-Jones, unpublished data). Moreover, monoclonal antibodies against the toxin may be used in frozen section to monitor mycolactone level and distribution to indirectly measure viability of *M. ulcerans* in the course of therapy. Both methods could potentially also be suitable as diagnostic tools performed in reference laboratories.

In leprosy, a reverse transcription (RT)-PCR assay targeting 16S rRNA of *M. leprae* has been used to detect specific RNA in biopsy specimens [34]. Positive results were obtained in biopsies from fresh or short term treated cases. In contrast, from specimens of long term treated cases showing clinical features of relapse were

negative. DNA targeting PCR showed positivity in both groups. These results suggest that RT-PCR positivity possibly reflects the presence of viable organisms and that a proportion of supposed relapses may rather appear as secondary immune "reactivation" due to remaining dead *M. ulcerans*. Thus RT-PCR assays may be useful for viability determinations of *M. ulcerans* in response to chemotherapy as well as in supposed relapse cases.

12.3.4. Vaccine development

Prevention of clinical disease, as provided by vaccination, is the most desired solution to finally control *M. ulcerans* infection. Studies whether BCG vaccination may render herd immunity as it is found for leprosy [35] were not overly successful [36-38], although severe disseminated infections may be prevented [39]. Other approaches based on DNA engineering and virulence factors of *M. ulcerans* are under evaluation.

For the rational design of a vaccine it is important to identify the immune effector functions relevant for a particular infection. In the case of the best studied mycobacterial disease TB cellular immune responses are thought to be most crucial. Effective immune response against the primarily intracellular pathogen *M. tuberculosis* requires several subsets of immunity. The induction of CD4+ Th-1 cells is protective in mice [40] and is mirrored in humans with HIV co-infection who have a 500-fold increased risk of TB due to their reduced CD4+ leukocyte counts [41]. Furthermore, CD8+ cytotoxic T cells can recognise and destroy target cells infected with intracellular *M. tuberculosis* [42,43]. Mouse models of latent TB indicate that CD8+ T lymphocytes are particularly important in the prevention of reactivation [44]. Another, though not very popular, hypothesis argues that mycobacteria-specific antibodies induced by BCG vaccination could target extracellular *M. tuberculosis* implicated in transmission and actively replicating within cavitary pulmonary lesions [45].

In *M. ulcerans* infection leukocyte subsets required for immunity are supposed to be different as replication happens predominantly extracellular. Intracellular *M. ulcerans* are released from phagocytes due to the action of mycolactone and eliciting specific CD4+ Th-1 and CD8+ T cells may play a minor role in the containment of infection. Therefore, the humoral immune response potentially represents the most important

effector arm of the immune system against *M. ulcerans*. In addition, eosinophils and mast cells may play an important role in immunity against the pathogen. This hypothesis is supported by results from antibiotic treated lesions where eosinophils gather around infected areas (see Chapter 3 and 5).

M. marinum is thought to be the common ancestor of all *M. ulcerans* strains [46]. BCG derived from *M. bovis* may lack proteins crucial for protection against BU [36,38]. A recombinant BCG strain over-expressing key *M. ulcerans* antigens might be a promising approach to enhance the vaccine's potency. However, standard BCG vaccination can induce prolonged ulcerative lesions at the site of intradermal inoculation which might even be enhanced through expression of *M. ulcerans* proteins. Especially immuno-compromised individuals may suffer from severe infectious complications [47]. An attenuated *M. ulcerans* strain deprived of mycolactone production which can persist only for limited periods *in vivo* but carries all relevant antigens may represent an alternative. In guinea pigs, mycolactone negative *M. ulcerans* strains have been shown to trigger a brisk inflammatory response which is limiting the experimental infection [18]. Under-attenuation could result in vaccine-associated BU disease whereas over-attenuation would greatly reduce immunogenicity.
A strategy that avoids risks associated with a life vaccine is the utilization of whole killed *M. ulcerans* to introduce antibody responses against its immunodominant antigens. However, this approach might provide rather low protection, because antibody responses against molecules with low immunogenicity may be primarily relevant.

Subunit vaccines, based on recombinant proteins or plasmid DNA, represent a much safer strategy. A major drawback for protein subunit vaccines is the insufficient availability of adjuvants to trigger antigen-presenting cells. To date, only alum and QS-21 are approved for use in humans. DNA vaccines offer the advantage of endogenous antigen production inside antigen-presenting cells and contain CpG motifs triggering appropriate responses via TLR9 signalling after vaccination [48]. However, attempts to use a *Mycobacterium leprae* Hsp65-based DNA vaccine against *M. ulcerans* infection in mice failed [49]. Recently, immunogenicity and protective efficacy of Ag85A from *M. tuberculosis* and *M. ulcerans*, administered as a

plasmid DNA vaccine, as a recombinant protein vaccine in adjuvant or as a combined DNA prime-protein boost vaccine, was reported [50]. Combination with a vaccine targeting mycolactone may be a way to strengthen the effectiveness of this potential subunit vaccine. Another market-approved adjuvant and carrier system are influenza virosomes [51,52]. Antigenic pathogen-specific peptides could be linked to virosomes to present *M. ulcerans* antigens to the immune system. Alternatively, synthesized mycolactone could be hooked to the virosomal surface to generate immunity against the cytotoxin.

Even if future research will yield promising results with vaccine candidates in the animal model it will be hard to raise enough money to implement clinical trials for BU vaccine development despite all efforts in rising awareness of the disease. An additional opportunity is to wait for the development of an improved TB vaccination which may evoke cross-reactive immune responses providing protection against *M. ulcerans* infection.

12.4. Future aspects of disease control

One focus to intensify BU control activities lies on the education of the population in endemic countries which would improve case management through early case detection at community and school level. Patients need to be aware of the early symptoms and their confidence in the available treatment options has to be strengthened. Many people believe in witchcraft as the main factor to develop such diseases and prefer traditional healers over classical medicine [53], so the true cause of BU has to be made clear to those affected. In a study in Benin presentation delay decreased from four months in 1989 to one month in 2001 due to continuing efforts to raise the population's education and awareness for the disease [54].

A decentralization of health care could help to reach people in the very remote rural areas who will not present to a hospital because of the great distance [53]. Village health workers need to be trained to recognize and treat the disease, especially when a fully orally administered antibiotic regimen or thermotherapy is available. Diagnostic centres are needed in the periphery, as rural areas suffer from the inability

to diagnose *M. ulcerans* infection in a timely manner, especially to unambiguously diagnose closed BU lesions which lack the pathognomonic features of ulcerative lesions. Synergies with other disease-control activities using already existing networks and infrastructures could help to accelerate implementation of these issues. It is important not only to create facilities but also to assure sustainability and a continuum of care by strengthening national health systems with the contribution of NGOs and thereby guaranteeing provision of equipment and resources.

Correct treatment of BU includes a third essential element apart from surgery and drugs, namely prevention of disability (POD). Reduction in the range of motion or other more severe functional limitations of joints are commonly observed to accompany chronic *M. ulcerans* infections [55] and have significant impact on employment or schooling and farming activities [56]. Joint contractures often have to be removed surgically, but also aftercare physiotherapy has been shown to improve disabilities [57]. Some health centres are already equipped with a physiotherapy unit including trained personnel and special equipment, but all caregivers should receive basic training in POD. WHO is currently working on a POD manual within its series of training modules on BU which will be targeted at general health staff [28].

West Africa and other developing countries endemic for BU will never be able to cover all communities with high-standard resources, but every small improvement is one step further to diminish suffering among affected individuals. General practitioners could be educated, for instance, to perform nodulectomies or simple split skin grafts, because workload is already too high in most regions to exclusively rely on fully-trained surgeons. Only ten years ago BU was an obscure disease to many living in endemic regions and even totally unacquainted on international level, and research was an extremely tedious business. Nowadays we are witnessing growing interest in this particular of defined 13 neglected tropical diseases worldwide [58], possibly leading towards an accelerated progress within the ten years to come.

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APPENDIX

Detection of anti-rec18kD antibodies in human serum samples using Western blot analysis

Daniela Schütte, Diana Díaz-Arévalo*, Gerd Pluschke

Swiss Tropical Institute, Section Molecular Immunology, Basle, Switzerland

*Current address: City of Hope, Duarte, CA, USA

A large range of serum samples collected in Ghana, Cameroon and Australia was tested for specific antibody response against the 18kD shsp in a Western Blot analysis to see whether infection with or exposure to M. ulcerans could be detected as previously described for Ghana [1]. For each country 13 patient sera were tested on recombinant 18kD protein for specific antibody responses. All 13 sera from Ghana (100%) were strong positive whereas only 5 out of 13 samples (38%) from Cameroonian patients showed an intermediate and 8 (62%) an extremely weak response which was (Fig.1). In the Australian set of patient sera merely 2 out of 13 sera (23%) yielded a faint positive and 5 (38%) a nearly invisible result (Fig.1)
Sera from individuals living in non-endemic countries showed no response to the 18kD protein at all [1]. We collected sera from non-endemic regions of BU endemic countries and tested those for their anti-18kD antibody response in comparison to patient sera. 12 out of 30 patients sera (40%) from an endemic BU area in Cameroon showed a positive response to the recombinant 18kD protein (Fig.2a). 13 out of 36 sera (36%) from a BU non-endemic neighbouring region in Cameroon were positive for anti-18kD antibodies (Fig.2b). A similar result was obtained for sera retrieved from a BU non-endemic area located in the dry northern Ghana, where 13 out of 42 samples (31%) were Western Blot positive (Fig.2c). Finally, 13 out of 20 samples (65%) collected in a BU non-endemic village located inside the Savannah of northern Cameroon with a high leprosy incidence rate gave a positive anti-18kD response (Fig.2d).

In a previous work we could show that the recombinant 18kD is recognized by Ghanaian sera of BU patients and household contacts, therefore identifying exposure to the pathogen [1]. Surprisingly, this was not the case for sera collected in Cameroon or Australia where percentage of patient samples recognizing the 18kD protein was less than in Ghana and positive signals lower in intensity. The reason for this phenomenon is unclear, but host factors might play a role as well as severity and location of the lesion. When around a third of sera from non-endemic regions within Cameroon and Ghana turned out positive, cross-reactivity with another protein became conceivable. On this account, sera from a region of Cameroon non-endemic for BU but with increasing prevalence of leprosy were tested and 65% showed reaction against the recombinant 18kD shsp of *M. ulcerans*. *M. leprae* carries a homologue of 18kD shsp with 78.8% protein sequence identity and it is likely that

individuals infected or exposed to this pathogen elicit a similar immune response as against *M. ulcerans* exposure [1,2]. This would also explain the very low reactivity in sera collected in Australia, where leprosy is not endemic.

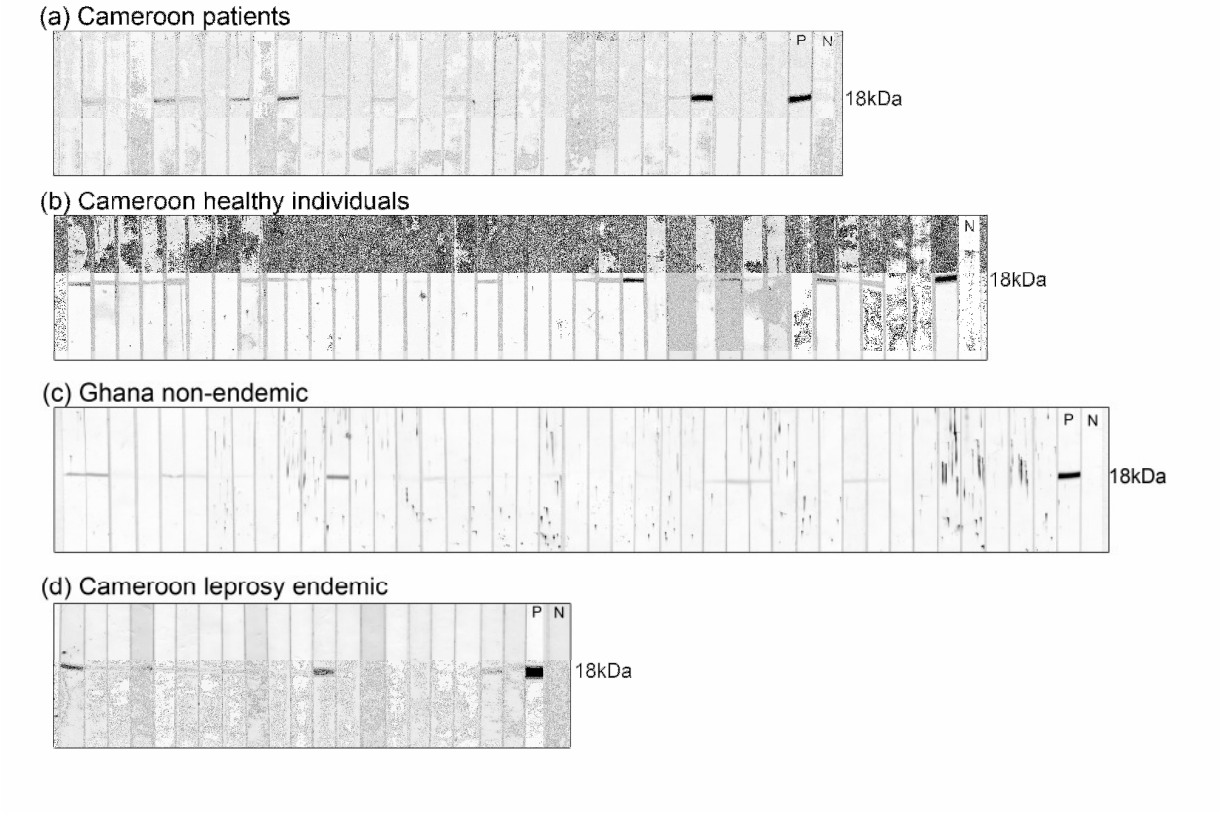

Figure1. Western blot analysis of sera from BU patients and individuals living in non-endemic regions

SDS-page gels loaded with recombinant 18kD protein were blotted to nitrocellulose membranes and cut into 3mm strips. Strips were incubated with sera samples coming from different African regions. Individuals with confirmed BU disease (a), healthy individuals living in Buruli non-endemic areas of Cameroon (b) and Ghana (c) and individuals from a village non-endemic for BU but with a high prevalence of leprosy (d). Positive control (P) with monoclonal antibody and negative control with skin milk in PBS-Tween alone.

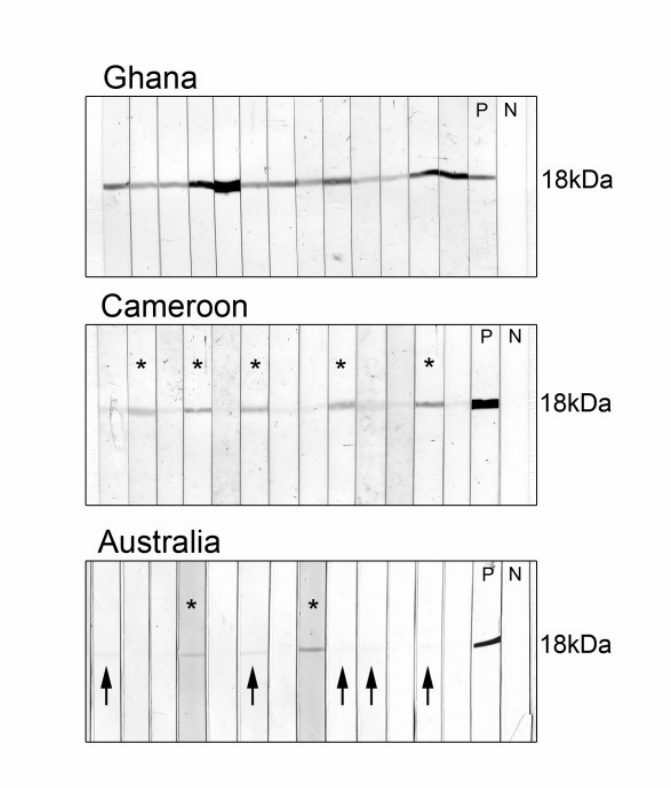

Figure2. Western blot analysis of sera from BU patients of three different countries

SDS-page gels loaded with recombinant 18kD protein were blotted to nitrocellulose membranes and cut into 3mm strips. Strips were incubated with sera samples taken from Ghanaian, Cameroonian and Australian BU patients. Positive control (P) with monoclonal antibody and negative control with skin milk in PBS-Tween alone.

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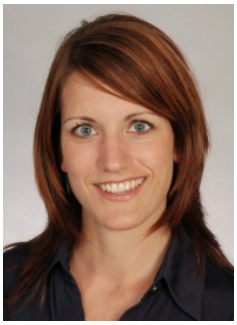
CURRICULUM VITAE



DANIELA SCHÜTTE

Riehenstrasse 62, CH – 4058 Basel

phone: 0041-78-8789006, email: daniela.schuette@unibas.ch

PERSONAL DATA

Date of birth:	05.10.1978
Place of birth:	Mayen/ Koblenz
Nationality:	German
Marital status:	Single

EDUCATION

05/05–09/08 **PhD thesis (Microbiology), Grade: magna cum laude**
Approaches to improve treatment and early diagnosis of Buruli ulcer: the role of local and systemic immune responses
Swiss Tropical Institute, Basel

06/03–12/03 **Diploma thesis (Virology), Grade: 2.0**
Centre of Genetic Research, Munich

10/98–05/03 **Major: Biology, Grade: 1.5**
Technical University Munich (TUM)

09/88–06/97 **Allgemeine Hochschulreife, Grade: 2.1**
Gymnasium Starnberg (grammar and high school)

WORK EXPERIENCE/ EMPLOYMENT ABROAD

11/05 **Visiting researcher, TB unit** *(antimycobacterial drug screening)*
Novartis Institute for Tropical Diseases (NITD), Singapore

01/04–08/04 **Work experience, Medical Research Unit** *(Malaria research)*
Albert Schweitzer Hospital, Lambaréné, Gabon

06/04 **Internship, surgical ward** *(rounds and theatre)*
Albert Schweitzer Hospital, Lambaréné, Gabon

02/02–04/02 **Internship, wolf farm "Lobopark"** *(behavioural studies)*
Antequera, Malaga

02/01–02/02 **Work experience, Ingenium AG** *(ENU mouse mutagenesis project)*
Department for Radiation Research (GSF), Munich

CONGRESSES

31/03-03/04/08	**Presentation** *("Phagocytosis of Mycobacterium ulcerans in the course of rifampicin and streptomycin chemotherapy")* 10th Annual meeting of the Global Buruli Ulcer Initiative (GBUI) World Health Organization (WHO), Geneva
17/03-19/03/08	**Presentation** *("Immunohistological evaluation of local cellular immune responses in Buruli ulcer lesions after antibiotic treatment")* 20th Meeting of the Swiss immunology PhD students Schloss Wolfsberg, Ermatingen
04/12-05/12/07	**Presentation & Chair** *("Immunohistological evaluation of local cellular immune responses in Buruli ulcer lesions after antibiotic treatment")* SSTMP PhD student meeting, Münchenwiler
23/10/07	**Poster** *("Local Cellular Immune Response Evolving Due to Antibiotic Therapy in Buruli Ulcer Lesions")* BioValley Science Day, Basel
18/10-20/10/07	**Poster** *("Local Cellular Immune Response Evolving Due to Antibiotic Therapy in Buruli Ulcer Lesions")* 89th Annual meeting of the Swiss Society for Dermatology and Venerology (SGDV), Bern
24/05-28/05/07	**Presentation** *("Immunohistological evaluation of antibiotic treatment with rifampicin and streptomycin")* 5th European Congress on Tropical Medicine and International Health, Amsterdam
01/04-04/04/07	**Presentation** *("Immunohistological evaluation of host responses to antibiotic treatment with rifampicin and streptomycin")* 9th Annual meeting of the Global Buruli Ulcer Initiative (GBUI) World Health Organization (WHO), Geneva

TECHNIQUES

Histology	standard staining, immunohistochemistry, fluorescence, microscopy
Drug screening	toxicity test, growth inhibition assay
Cell culture	transfection, monoclonal antibody production (hybridoma technology)
Basic techniques	cloning, blots, PCR, ELISA
Blood sampling	PBMC separation, culture, thick smear, CellDyn 2000
Special	mouse experiments, helminth screening, working in S3-facilities, planning and conducting studies in Africa

PUBLICATIONS

In preparation	**J Invest Dermatol** Schütte D, et al. *Heat treatment of Buruli ulcer is not associated with massive local immune activation*

In preparation	Schütte D, et al. *Compound DS-1 effectively inhibits growth of in vitro cultured Mycobacterium ulcerans*
In preparation	Schütte D, et al. *Diagnosis of Mycobacterium ulcerans infection by its 18kD small heat shock surface protein*
In preparation	**PLoS Negl Trop Dis** Schütte D, et al. *Local immune responses and bacterial killing in early stage Buruli ulcer lesions during treatment with rifampicin and streptomycin*
2009	**Exp Opinion Biol Therapy (review)** Schütte D, Pluschke G. *Immunosuppression and inflammatory response in patients with Buruli ulcer*
2009	**PLoS Negl Trop Dis** Junghanss T, Um Boock A, Vogel M, Schütte D, et al. *Phase change material for thermotherapy of Buruli ulcer*
2008	**Brit J Dermatol** Schütte D, et al. *Phagocytosis of the extracellular pathogen Mycobacterium ulcerans in the course of rifampicin and streptomycin chemotherapy*
2007	**PLoS Negl Trop Dis** Schütte D, et al. *Development of highly organized lymphoid structures in Buruli ulcer lesions after treatment with rifampicin and streptomycin*
2007	**J Invest Dermatol** Peduzzi E, Groeper C, Schütte D, et al. *Local activation of the innate immune system in Buruli ulcer lesions*
2005	**Am J Trop Med Hyg** Adegnika AA, Breitling LP, Agnandji ST, Chai SK, Schütte D, et al. *Effectiveness of quinine monotherapy for the treatment of Plasmodium falciparum infection in pregnant women in Lambaréné, Gabon*

ADDITIONAL QUALIFICATION

Languages	German (native speaker) English (fluently written and spoken) French (basic knowledge) Italian (basic knowledge) Latin (Latinum)
Software	Windows, Internet, Microsoft Office (Word, PowerPoint, Excel), Photoshop, CorelDraw, FileMaker, EndNote, Reference Manager
Interests	Sports (JiuJitsu, Rowing, Aerobics, Skiing), Travelling, Reading

creative commons

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