Am. J. Trop. Med. Hyg., 73(2), 2005, pp. 410–415

HIGH RATES OF APOPTOSIS IN HUMAN *MYCOBACTERIUM ULCERANS* CULTURE-POSITIVE BURULI ULCER SKIN LESIONS

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Abstract. Buruli ulcer, a disease caused by *Mycobacterium ulcerans*, causes ulcerative skin disease likely generated by a toxin that mediates apoptosis. We analyzed paraffin-embedded sections of surgically excised Buruli ulcer lesions (two ulcers and one edematous plaque) and adjacent non-lesional skin samples (n = 9) for apoptosis by an indirect immunofluorescent terminal deoxynucleotide transferase–mediated dUTP-digoxigenin nick end labeling (TUNEL) assay. All samples were stained for acid-fast bacilli (AFB) and cultured for mycobacteria, and most were analyzed with an *M. ulcerans*-specific diagnostic polymerase chain reaction (PCR). TUNEL (+) bodies were numerous in both ulcers and the plaque, and sparse or absent in adjacent non-lesional skin. The AFB tissue stains and cultures for *M. ulcerans* were positive only in the three lesions. The result of the PCR for *M. ulcerans* was positive in all three lesions and in four of six non-lesional tissue samples; three contained sparse TUNEL (+) bodies. An abundance of TUNEL (+) bodies in the three AFB stain (+), culture (+), and PCR (+) Buruli ulcer lesional samples, but not in nearby AFB stain (−), culture (−), and PCR (+) non-lesional skin samples, strengthen the evidence that apoptosis is an important tissue destruction mechanism in human lesions closely associated with viable *M. ulcerans*.

INTRODUCTION

Buruli ulcer is a potentially destructive infection caused by *Mycobacterium ulcerans* that generally affects the skin and proximal underlying structures.[1] In severe cases, the disease may involve 20% or more of a person's skin surface, and up to 15% of patients have bone involvement.[1] The disease typically starts as a painless papule that may go unnoticed, most commonly on the extremities, and may then expand into a plaque or widespread indurated edema and subsequently ulcerate.[2] The ulcer may slowly enlarge, exposing underlying structures such as tendons or bone, but there is remarkably little pain and only limited histologic evidence of cellular infiltration, especially in advanced stages of the disease.[2,3] Medical treatment of progressive lesions is generally unsatisfactory, usually requiring wide surgical excision and skin grafting for cure.[4]

Most pathogenic bacteria produce toxins that mediate pathogenicity. For the two most common pathogenic mycobacteria, *M. tuberculosis* and *M. leprae*, no toxin has yet been identified. For Buruli ulcer, the third most common mycobacterial infection in humans, earlier work described a lipid-like toxin in cell-free culture filtrates of *M. ulcerans* that is cytotoxic and, upon skin inoculation, causes lesions in laboratory animals similar to human infections.[5–7] More recently, a growing body of evidence suggests that an important mechanism by which *M. ulcerans* causes massive tissue destruction with a disproportionate lack of acute inflammation, unlike *M. tuberculosis* and *M. leprae*, is toxin-mediated apoptosis.[2,8–11] George and others isolated a compound from *M. ulcerans* known as mycolactone, a polyketide-derived macrolide that is highly cytotoxic, which causes apoptosis in cell monolayers *in vitro* and human-like features of Buruli ulcer in guinea pig models.[12–14] Other work showed that mycolactone has immunomodulatory properties, blunting cytokine responses in T cells and monocytes and inhibiting NF-κB function.[15,16] To provide an *in vivo* correlate of apoptosis in human lesions, we examined surgically excised paraffin-embedded sections of well-characterized Buruli ulcer lesions and adjacent non-lesional skin samples using an immunoflourescent terminal deoxynucleotide transferase–mediated dUTP digoxigenin nick end labeling (TUNEL) assay that reliably detects apoptosis.[10]

MATERIALS AND METHODS

Adult (n = 5) and minor (n = 6) patients in Côte d'Ivoire or Benin electing surgical excision for Buruli ulcer gave informed consent or parental assent, respectively, for a general surgical procedure, acknowledging that excisonal lesion and adjacent non-lesional tissue samples might be used for research. Adjacent non-lesional samples were considered clinically normal appearing in the proximity of lesional skin that was removed during wide surgical excision. Lesional (n = 3; three patients) and adjacent non-lesional (n = 9; eight patients) samples were embedded in paraffin and stained with hematoxylin and eosin for routine histopathologic analysis and by the Ziehl-Neelsen method for acid-fast bacilli (AFB). Smears of homogenated tissue samples were stained with the Ziehl-Neelsen reagent and the amount of AFB observed in each sample was graded on a semi-quantitative scale as follows: 0 (none), 1+ (sparse), 2+ (scattered), or 3+ (abundant).[17] Parts of each sample were cultured on Löwenstein-Jensen media to assess for the presence of viable *M. ulcerans* and most samples were assessed by an *M. ulcerans*–specific IS*2404* polymerase chain reaction (PCR) as previously described.[18–20] Lesional material consisted of two ulcers and one edematous plaque from a patient with the disseminated form of Buruli ulcer.[21]

Assessment for apoptosis was conducted on formalin-fixed, 5-μM paraffin-embedded sections of lesional and adjacent

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non-lesional samples using a commercially available immunofluorescent TUNEL assay (kit S7110, ApopTag; Intergen Company, Purchase, NY). Briefly, sections were deparaffinized, digested with proteinase K (20 μg/mL in phosphate-buffered saline [PBS]; Sigma Chemical Company, St. Louis, MO) at room temperature for 15 minutes, and soaked in PBS for 5 minutes. Sections were then immediately incubated with working-strength terminal deoxynucleotide transferase in a humidified chamber at 37°C for one hour. The sections were immersed in stop buffer to terminate the enzymatic reaction and then gently rinsed with PBS. Fluorescein isothiocyanate (FITC)–labeled anti-digoxigenin conjugate was then applied to each section and incubated at room temperature for 30 minutes in the dark. Slides were washed in PBS, mounted with DABCO containing 0.5 μg/mL of propidium iodide, and visualized with a fluorescent microscope (Trinocular Photo-auto-micrographics BX51TRF; Olympus, Melville, NY) using an excitation wavelength of 460–490 nM. The TUNEL bodies, which are indicative of apoptosis, are readily identified as rounded, highly condensed, bright fluorescent structures.[10]

Paraffin-embedded, regressing rat mammary gland and smears of Jurkat cells (T cell leukemia cell line) treated with apoptosis-activating anti-Fas antibody were used as positive controls. Paraffin-embedded skin tag, a common benign human skin lesion, and smears of non-activated Jurkat cells were used as negative controls. In some experiments, primary or secondary antibodies were omitted to confirm the absence of non-specific staining. The entire area of each section processed for the TUNEL assay was viewed at 200× and the experimental samples were scored in a blinded, semi-quantitative fashion for the presence of TUNEL (+) bodies as none (0), sparse (1+), scattered (2+), or numerous (3+), as previously described.[10,22]

In an attempt to characterize the TUNEL (+) bodies by dual staining, some sections were chosen for fluorescent immunophenotypic or vimentin staining before undergoing TUNEL analysis. Vimentin is a cytoskelatal intermediate filament present in fibroblasts and endothelial cells.[23] Antibodies for immunophenotypic analyses included anti-CD3 (T cells; polyclonal) and anti-CD68 (macrophages; monoclonal, clone KP1); both were obtained from DakoCytomation (Carpinteria, CA). Vimentin antibody (clone V9) was obtained from Novocastra Laboratories, Ltd., Newcastle upon Tyne, United Kingdom). After deparaffinization, sections were processed for antigen retrieval. This included microwaving for anti-CD68 and vimentin and pressure cooking for anti-CD3. Individual sections were then incubated with unlabeled primary antibody (one hour at room temperature for vimentin, and overnight at 4°C for anti-CD3 and anti-CD68). Antibody dilutions for staining experiments were 1:400 for anti-CD3, 1:100 for anti-CD68, and 1:50 for vimentin. The sections were then washed with PBS-Tween buffer and reacted with Texas Red (Molecular Probes, Inc., Eugene, OR), a red fluorophore that has little spectral overlap with green fluorescein. For all experiments, Texas Red was diluted 1:400 with PBS and placed on the sections for 30 minutes at 37°C. The sections were washed with PBS-Tween and then processed for TUNEL reactivity as described above. The sections were mounted with DABCO and assessed under a multiple color fluorescent microscope (Trinocular Photoautomicrographics BX51TRF; Olympus) by alternating excitation wavelength ranges of 460–490 nM and 510–550 nM for FITC and Texas Red, respectively, which allow simultaneous visualization of double-positive structures, as previously described.[23] Before assessing the Buruli ulcer samples, staining of paraffin-embedded inflammatory skin lesions and lymphoid tissue was conducted with anti-CD3, anti-CD68, and vimentin to establish reproducible conditions. Anti-CD3 and anti-CD68 were also routinely assessed on peripheral blood smears to ensure antibody integrity.

TABLE 1

Patient characteristics, TUNEL staining, and other laboratory assessments of Buruli ulcer lesional and adjacent non-lesional samples and control samples*

Patient age (years) and sex	Sample and description	Culture for *Mycobacterium ulcerans*	Tissue AFB stain†	PCR for *M. ulcerans*	TUNEL assay‡
Buruli ulcer lesional samples					
9/F	Ulcer	+	3+	+	3+
29/F	Ulcer	+	3+	+	3+
9/M	Edematous plaque	+	2+	+	2+
Buruli ulcer non-lesional samples					
12/M	Skin adjacent to lesion	–	0	+	1+
1/F	Skin adjacent to lesion	–	0	+	1+
7/M	Skin adjacent to lesion	–	0	+	1+
60/M	Skin adjacent to lesion	–	0	+	0
40/F	Skin adjacent to lesion	–	0	–	0
42/F§	Skin adjacent to lesion	–	0	NT	0
42/F§	Skin adjacent to lesion	–	0	NT	0
17/M	Skin adjacent to lesion	–	0	NT	0
60/M	Skin adjacent to lesion	–	0	–	0
TUNEL assay controls					
	Cell (+) control: activated Jurkat cells	NA	NA	NA	2+
	Tissue (+) control: mouse mammary gland	NA	NA	NA	2–3+
	Cell (–) control: untreated Jurkat cells	NA	NA	NA	0
	Tissue (–) control: human skin tag	NA	NA	NA	0

* See Materials and Methods for details of both grading systems. TUNEL = terminal deoxynucleotide transferase–mediated dUTP-digoxigenin nick end labeling; AFB = acid-fast bacilli; PCR = polymerase chain reaction; + = positive; – = negative; NT = not tested; NA = not applicable.
† Ziehl-Neelsen AFB stain graded as 0 to 3+ for the amount of AFB.
‡ Graded as 0 (none); 1+ (sparse); 2+ (scattered); 3+ (numerous).
§ Same patient.

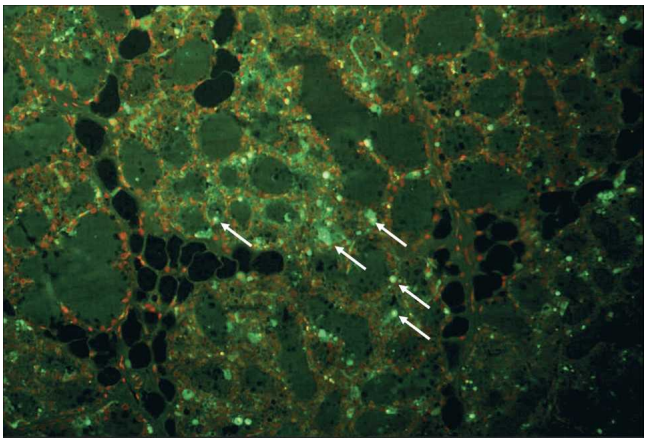

FIGURE 1. Regressing rat mammary gland as a terminal deoxynucleotide transferase–mediated dUTP-digoxigenin nick end labeling (TUNEL) assay (+) control showing many rounded, condensed, highly fluorescent TUNEL (+) bodies (**arrows**), some in clumps, consistent with apoptosis (original magnification × 400).

RESULTS

In all experiments, positive control sections of regressing rat mammary gland and activated Jurkat cell smears showed prominent, compact, brightly fluorescent TUNEL (+) bodies consistent with apoptoses (Figure 1). The human skin tag samples used as negative controls were negative for TUNEL bodies in all experiments.

The characteristics of the patients with Buruli ulcer and the results of the TUNEL assay, tissue AFB stain, mycobacterial culture, and diagnostic PCR of the three lesional samples and nine adjacent non-lesional tissue samples are summarized in Table 1. Among the three Buruli ulcer lesional samples, the two ulcers had numerous TUNEL (+) bodies, in some areas appearing more abundant than the positive control sample (Figure 2, left). The third lesion, an edematous plaque, had scattered TUNEL (+) bodies. Most TUNEL (+) bodies were located in the deep dermis and upper subcutaneous fat regions where widespread cell-poor necrosis and many AFB were observed (Figure 2, right, upper and lower images, respectively). All three lesion samples were AFB stain (+), *M. ulcerans* PCR (+), and on culture grew *M. ulcerans*.

Buruli ulcer adjacent non-lesional samples showed small numbers (n = 3) or complete absence (n = 6) of TUNEL (+) bodies (Figure 3, left). Unlike lesional samples, the non-lesional samples generally had modest cellular infiltrates in the dermis and subcutaneous fat with minimal necrosis (Figure 3, right). None of the samples were AFB stain (+) or grew *M. ulcerans* on culture. The PCR showed that four of six samples were conclusive for *M. ulcerans*, three of which contained small numbers of TUNEL (+) bodies.

Dual staining experiments in a lesional sample using vimentin and TUNEL showed scattered structures with red and green staining positivity, suggestive of vimentin (+)/TUNEL (+) cells (Figure 4). In lesional samples processed for CD3 or CD 68 and then TUNEL, none contained dual stained structures suggestive of CD3 (+)/TUNEL (+) or CD68 (+)/TUNEL (+) cells.

DISCUSSION

We show that human lesions of Buruli ulcer are characterized by high levels of apoptosis. Apoptoses were most promi-

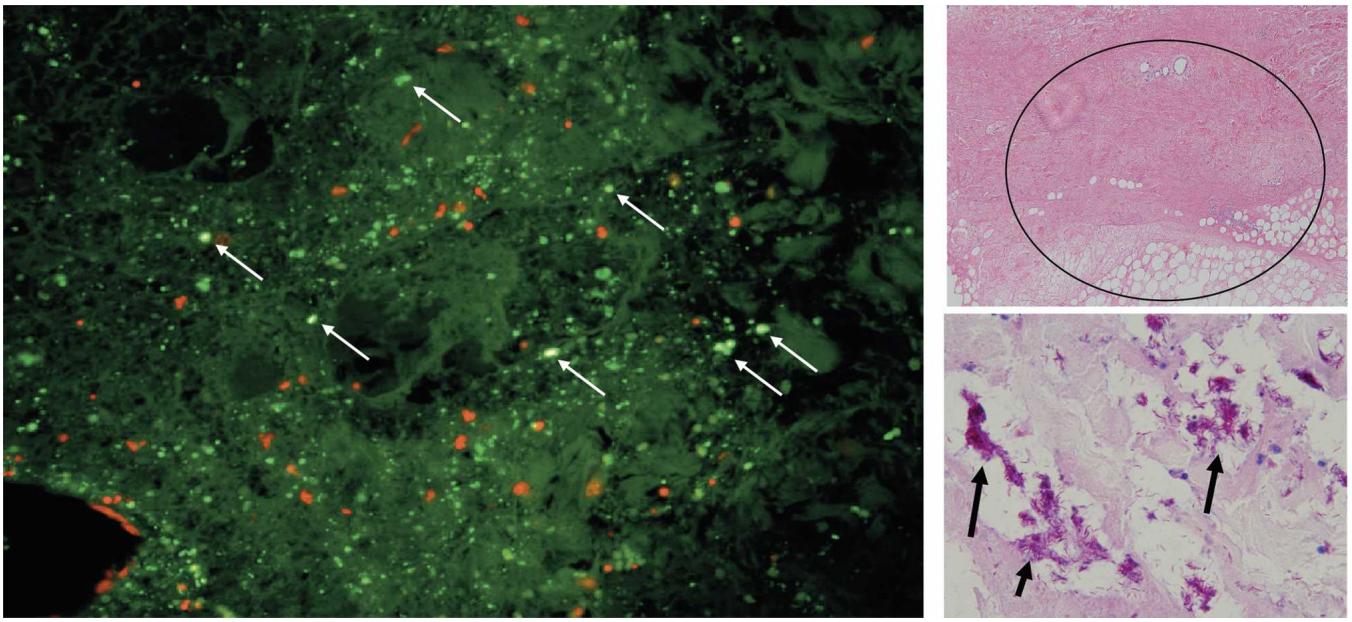

FIGURE 2. **Left**, Representative section of a Buruli ulcer lesional sample processed for terminal deoxynucleotide transferase–mediated dUTP-digoxigenin nick end labeling (TUNEL) showing abundant (3+) TUNEL (+) bodies in the deep dermis, some in clumps (**arrows**) (original magnification × 400). This sample was acid-fast bacilli (AFB) stain (+) (see image lower right), *Mycobacterium ulcerans* culture (+), and *M. ulcerans* polymerase chain reaction (+) **Upper right**, Hematoxylin and eosin staining of the same Buruli ulcer lesional sample showing extensive, deep dermal and fat necrosis with minimal cellular infiltrate (**oval**), consistent with an advanced lesion (original magnification × 20). **Lower right**, Ziehl-Neelsen AFB staining of same Buruli ulcer lesional sample showing abundant extracellular AFB (**dark red**), some in clumps (**arrows**) (original magnification × 1,000).

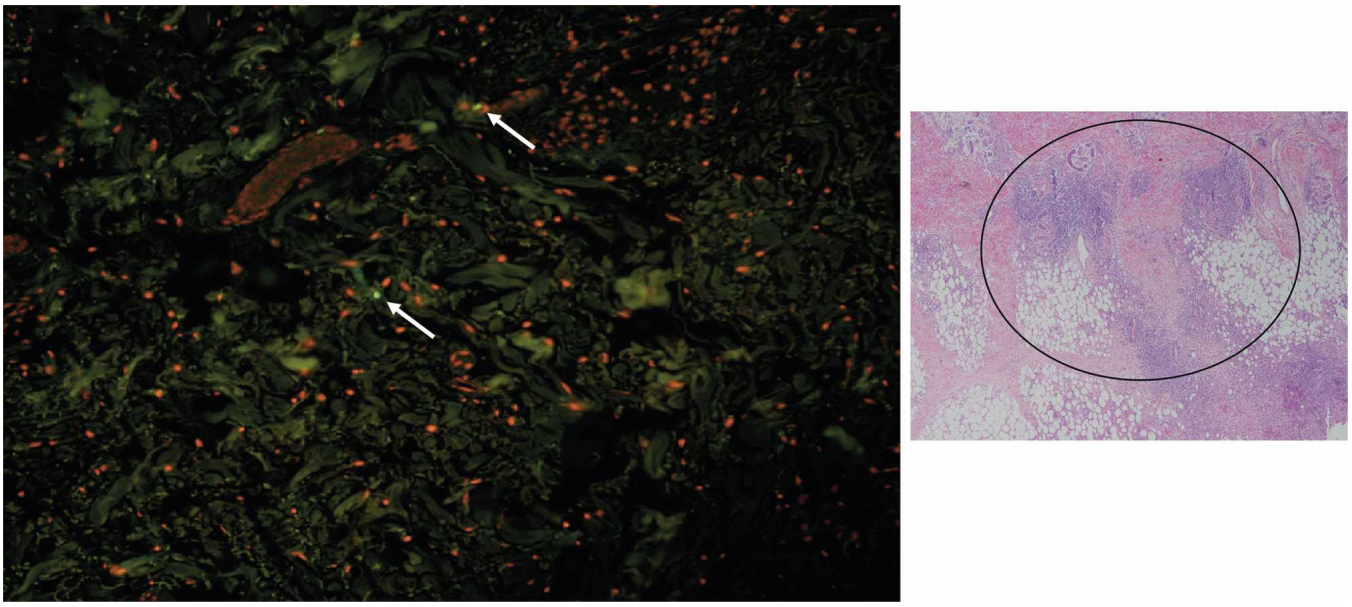

FIGURE 3. **Left**, Representative section of a non-lesional skin sample adjacent to a Buruli ulcer lesion processed for terminal deoxynucleotide transferase–mediated dUTP-digoxigenin nick end labeling (TUNEL) showing sparse (1+) TUNEL (+) bodies in the deep dermis (**arrows**) (original magnification × 200). This sample was acid-fast bacilli stain (–), *Mycobacterium ulcerans* culture (–), and *M. ulcerans* polymerase chain reaction (+). **Right**, Hematoxylin and eosin staining of same Buruli ulcer adjacent non-lesional skin sample showing minimal dermal and fat necrosis and a modest cellular infiltrate (**oval**), consistent with an early lesion (original magnification × 40).

nent in necrotic areas of the deep dermis and fat layers of the three lesional samples, which were composed of two ulcers from two different patients and one edematous plaque, which represented a disseminated form of Buruli ulcer not typically associated with ulceration.[21] This suggests the pathogenic factors of Buruli ulcer related to apoptosis are similar, regardless of lesion morphology. Notably, the three lesion samples, but none of the adjacent non-lesional samples, also contained AFB and on culture grew *M. ulcerans*. In contrast, non-lesional tissue adjacent to the lesions, some of which were PCR (+) for *M. ulcerans*, showed far fewer TUNEL (+) bodies and were all AFB (–) and culture (–). This observation indicates that apoptosis is a significant event in lesions of Buruli ulcer and that it is closely associated with viable organisms.

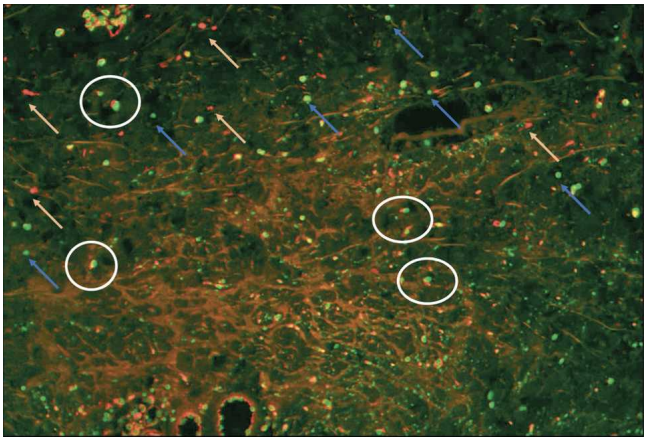

FIGURE 4. Buruli ulcer lesion sample shown in Figure 1 processed for vimentin (**red**) and terminal deoxynucleotide transferase–mediated dUTP-digoxigenin nick end labeling (TUNEL) (**green**). There are scattered vimentin (+) cells (**white arrows**) and TUNEL (+) bodies (**blue arrows**). Occasional double-stained rounded structures (**white ovals**) are suggestive of vimentin (+)/TUNEL (+) cells (original magnification × 200).

Secreted mycolactone from *M. ulcerans* is generally considered diffusible, based on histologic data of Buruli ulcer whereby necrosis occurs not only near *M. ulcerans* organisms, typically in extracellular colonies, but also extending some distance from the organisms.[3] Here, the distance between lesional and non-lesional skin samples was not recorded, but positive PCR results for *M. ulcerans* in four of six tested adjacent non-lesional samples, three of which showed small numbers of apoptoses, supported the notion that DNA from dead organisms, and likely the toxin that mediates apoptosis, diffused into adjacent deep dermis and subcutaneous regions beyond clinically evident lesions. In accordance with earlier work in leprosy lesions suggesting that detection of even a single apoptotic body in a 5-μM tissue section is consistent with appreciable cell death in the sample,[24,25] the high number of TUNEL bodies in the Buruli ulcer lesional samples indicated massive amounts of apoptosis.

Apoptosis, a physiologic process of individual cell death, is characterized by distinct morphologic and biochemical criteria that eliminates compromised or superfluous cells.[26] Unlike lysosomal-mediated cell death that generally involves acute inflammation and chemical mediators, apoptosis involves cell membrane asymmetry (blebbing), followed by nuclear fragmentation and condensation detectable by TUNEL analysis.[27,28] Growing evidence suggests apoptosis is relevant in some infectious diseases by regulating immune responses and, less commonly, by directly affecting microbial proliferation.[8,29] For tuberculosis (TB), and perhaps leprosy, apoptosis may be a consequence of an effective cellular immune response that assists in arresting intracellular bacterial

replication.[8–10,24,25,29] However, apoptosis in TB may be paradoxical, killing permissive infected macrophages and sequestering bacilli within apoptotic bodies or, less favorably, eliminating antigen-specific cytotoxic T cells destined to kill infected macrophages.[8,9,30] The latter characterizes highly virulent TB strains, which are mediated by over-expression of anti-apoptotic Fas ligand (FasL) on infected cells and establish a protective intracellular sanctuary.[9,30] In contrast, *M. ulcerans* is typically observed as an extracellular organism and, as our data show, confers abundant host tissue apoptosis.

Much work in Buruli ulcer has focused on toxin isolation from cultured organisms, especially mycolactone, which triggers apoptosis in cell monolayers and animal models.[12–14] Unlike apoptosis in TB or leprosy that is associated with notable inflammation,[9,10] apoptosis in Buruli ulcer occurs in necrotic regions with little inflammation. It remains unclear which cell types are targeted in Buruli ulcer apoptosis, but routinely stained sections showing deep dermal and subcutaneous destruction, with little or no cellular reaction, imply that at least fibroblasts and lipocytes may be primarily affected.[31] Some of our dual staining experiments suggested that TUNEL bodies in Buruli ulcer may contain vimentin, an important protein found in intermediate filaments of the cytoskeleton of cells such as fibroblasts and endothelial cells.[23]

Recognition that the pathogenesis of Buruli ulcer, a disease generally requiring surgical intervention for cure, involves large amounts of apoptosis may be useful in prophylactic or therapeutic approaches. For example, a vaccine that targets mycolactone, the likely cause of apoptosis, may be useful. Future work will focus on determining what cell types undergo apoptosis, and characterizing the expression of apoptotic-modulating molecules such as Fas, FasL, and tumor necrosis factor-α receptors in Buruli ulcer that may help predict the impact of medical therapies that affect these receptors.

Received February 10, 2005. Accepted for publication March 31, 2005.

Acknowledgments: We thank Sister Julia Aguiar and Dr. Lucie Avoaka-Cisse for providing the tissue samples, and Dr. Moo Hwang for photomicrography. This work was presented in part at the 53rd Annual Meeting of the American Society of Tropical Medicine and Hygiene, November 7–11, 2004, Miami, Florida (Abstract # 597). The American Committee on Clinical Tropical Medicine and Travelers' Health (ACCTMTH) assisted with publication expenses.

Financial support: This work was supported in part by the Damien Foundation (Brussels) and the American Registry of Pathology (Washington, DC).

Disclaimer: The opinions or assertions presented herein are the private views of the authors and are not to be construed as official or as reflecting the views of the U.S. Departments of the Army or Defense.

Disclosure: There are no known commercial interests or other associations that might pose a conflict of interest.

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