Use of the Immunodominant 18-Kilodalton Small Heat Shock Protein as a Serological Marker for Exposure to *Mycobacterium ulcerans*\(^{\dag}\)

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While it is well established that proximity to wetlands is a risk factor for contracting Buruli ulcer, it is not clear what proportion of a population living in an area where the etiologic agent, *Mycobacterium ulcerans*, is endemic is actually exposed to this disease. Immunological cross-reactivity among mycobacterial species complicates the development of a specific serological test. Among immunodominant proteins recognized by a panel of anti-*M. ulcerans* monoclonal antibodies, the *M. ulcerans* homologue of the *M. leprae* 18-kDa small heat shock protein (shsp) was identified. Since this shsp has no homologues in *M. bovis* and *M. tuberculosis*, we evaluated its use as a target antigen for a serological test. Anti-18-kDa shsp antibodies were frequently found in the sera of Buruli ulcer patients and of healthy household contacts but rarely found in controls from regions where the infection is not endemic. The results indicate that only a small proportion of *M. ulcerans*-infected individuals contract the clinical disease.

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. After tuberculosis and leprosy, Buruli ulcer is the third most common mycobacterial infection in immunocompetent humans. The main burden of disease falls on children living in sub-Saharan Africa, but healthy people of all ages, races, and socioeconomic class are susceptible as well (2). Buruli ulcer has been reported in over 30 countries, mainly those with tropical and subtropical climates, but it may also occur in some countries 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, but the numbers of cases reported in some countries of West Africa are substantial (5,700 cases between 1989 and 2003 in Benin, 17,000 cases between 1978 and 2003 in Côte d’Ivoire, and 5,619 cases in 1999 in Ghana).

In Buruli ulcer lesions, clumps of extracellular acid-fast organisms surrounded by areas of necrosis are often found in subcutaneous fat tissue (19). *M. ulcerans* produces a family of macrolide toxin molecules, the mycolactones, which are associated with tissue destruction and local immunosuppression (13). In cell culture experiments, mycolactones produce apoptosis and necrosis in many human cell types (8, 14). The toxin appears to play a role in inhibiting the recruitment of inflammatory cells to the site of infection, which explains at least in part why inflammatory responses are poor in Buruli ulcer lesions (19). Downregulation of Th-1 responses may play a role in the progression of early Buruli ulcer disease (15–17, 34), but this may reverse in later stages (11). Intralesional influx of leukocytes and granulomatous responses in the dermis and panniculus has been reported in late stages of the disease (11, 18). Spontaneous healing can occur and is often accompanied by a conversion of the Burulin (*M. ulcerans* sonicate) skin test from negative to positive.

In spite of some degree of local and peripheral T-cell anergy, Buruli ulcer patients seem to be able to raise a humoral immune response against *M. ulcerans* antigens (15), and analysis of the serological responses to culture filtrate antigens of *M. ulcerans* has suggested that serological tests may be useful in the diagnosis and surveillance of the disease (9, 31). Broad antigenic cross-reactivity between mycobacterial species represents a major problem for the development of a serological test that is specific and sensitive enough to monitor immune responses against *M. ulcerans* in populations where exposure to *M. tuberculosis* and BCG vaccination is common.

We reasoned that the identification, recombinant expression, and immunological profiling of immunodominant proteins will provide target structures for analyzing protective immune mechanisms and for the development of a serological test suitable for detecting *M. ulcerans* exposure and/or disease. We describe here serological responses against a highly immunogenic 18-kDa small heat shock protein (shsp) of *M. ulcerans* which has no homologue in *M. bovis* and *M. tuberculosis*. Serological analysis indicates that this protein represents a suitable target antigen for monitoring exposure to *M. ulcerans*.

**MATERIALS AND METHODS**

**Mycobacterial isolates.** The mycobacterial species included in the present study are *M. abscessus* (ATCC 19797), *M. avium* subsp. *avium* (MAC101), *M. boehmianum* (clinical isolate), *M. bovis* biotype BCG (ATCC 35734), *M. chelonae* (DSM 43804), *M. fortuitum* (ATCC 49403), *M. gordonae* (Pasteur 14021.001), *M. haemophilum* (ATCC 29548), *M. intracellulare* (clinical isolate), *M. kansasii* (NCTC 10268), *M. leprae* (clinical isolate), *M. malmoense* (NCTC 11298), *M. marinum* (ATCC 927), *M. scrofulaceum* (Pasteur 14022.0031), *M. smegmatis* (Pasteur 14133.0001), *M. terrae* (clinical isolate), *M. tuberculosis (ATCC 27294), and *M. ulcerans* (clinical isolate).
M. tuberculosis (Pasteur 14001.0001), and M. leprae (kindly provided by P. J. Brennan). The M. ulcerans isolates of diverse geographical origin analyzed in the present study were from the Democratic Republic of Congo (5151), Angola (960657), Ghana (97-483), Argentina (ITM 5147), ITM 9550, and 94-1324), Mexico (ITM 5114), Malaysia (941328), French Guiana (ITM 7922), and Japan (ITM 8756). The mycobacteria were cultured as described previously (37).

Mycobacterial lysates and subcellular fractions. Mycobacterial cells were heat inactivated at 80°C for 1 h and suspended in phosphate-buffered saline (PBS) (50 mM sodium phosphate, 150 mM sodium chloride [pH 7.4]) containing 5% sodium dodecyl sulfate (SDS) and 1 mM phenylmethylsulfonyl fluoride, and 10 μg each of leupeptin and soybean trypsin inhibitor (Sigma, St. Louis, Mo.). A total of 200 μg of cell suspension was subjected to a bead beater (Mikro-Dismembrator; Braun Biotech International) treatment with 400 μl of 0.1-mm zirconia beads (BioSpec Products) at 2,300 rpm for 15 min. Beads and unbroken cells were removed by centrifugation at 10,000 × g for 10 min. The protein content of the lysate was quantified by using a BCA protein assay (Pierce).

For the preparation of subcellular fractions, 400 μg of heat-inactivated M. ulcerans cells was suspended in 3 ml of PBS containing 0.1% Tween 80 and the content of the lysate was quantified by using a BCA protein assay (Pierce).

FIG. 1. Western blot analysis of lysates of different mycobacterial species with MAbs DD2.5 (A) and DD3.7 (B). Lanes: 1, M. abscessus; 2, M. avium; 3, M. bohemicum; 4, M. fortuitum; 5, M. gordonae; 6, M. haemophilum; 7, M. intracellulare; 8, M. kansasi; 9, M. scrofulaceum; 10, M. malmoense; 11, M. lentiflavum; 12, M. smegmatis; 13, M. smegmatis; 14, M. terrae; 15, M. xenopi; 16, M. chelonae; 17, M. lepra; 18, M. ulcerans; 19, M. tuberculosis; 20, M. marinum; 21, M. bovis; 22, recombinant M. ulcerans 18-kDa shsp. Although no reactivity with M. marinum ATCC 927 was observed, the M. marinum strain used for the genome project harbors an 18-kDa shsp gene that is nearly identical to the M. ulcerans gene.

For the preparation of subcellular fractions, 400 μg of heat-inactivated M. ulcerans cells was suspended in 3 ml of PBS containing 0.1% Tween 80 and the protein content of the lysate was quantified by using a BCA protein assay (Pierce). The preparation for the analysis of human sera, 1 μg of recombinant 18-kDa shsp of M. ulcerans was separated on SDS–12% PAGE preparative gels and transferred as described above. Human sera diluted 1:100 were incubated with the antigen strips for 1 h. These were washed five times with either nonstrirer (0.15 M PBS [pH 7.2], 0.1% Tween 20) or stringent (0.3 M PBS [pH 7.2], 1% Tween 20) wash buffer. After incubation for 1 h with alkaline phosphatase-conjugated AffiniPure F(ab′)2 fragment goat anti-human immunoglobulin G (Jackson Immunoresearch Laboratories), BCIP-nitroblue tetrazolium (Bio-Rad) was used as a substrate.

Generation of MAbs. Hybridoma cell lines were generated as described previously (32) from mice immunized intraperitoneally three times with 20 μg of a lysate of the M. ulcerans strain 97-610 from Ghana formulated in MPL-TDM adjuvant (Sigma). Three days before cell fusion, mice received an intravenous booster injection with 20 μg of M. ulcerans lysate in PBS. Antibodies specific for M. ulcerans antigens were identified by enzyme-linked immunosorbent assay using Immunonol 4 plates (Dynes Technologies, Inc., Chantilly, Va.) coated with M. ulcerans lysate. From the panel of hybridomas generated, Western blot analysis identified three (designated DD2.5, DD2.6, and DD3.6), that secreted MAbs specific for an 18-kDa protein.

Partial purification and identification of the MAb DD2.5/3.6 reactive protein. A total of 300 mg of heat-inactivated M. ulcerans cells (weight) was washed once with 1% sodium sarcosylate, washed five times with PBS, and dissolved in 20 ml of 0.8 M urea. After centrifugation at 20,000 × g for 30 min, the supernatant was applied onto a RP-8 HPLC column (Nucleosil 300-5 C8). The column was washed with 0.1% trifluoroacetic acid in water and then eluted with a gradient of acetonitrile (gradient A). After separation on a SDS–12% PAGE gel, a band of an apparent molecular mass of 18 kDa was excised and digested with trypsin as described previously (12). For nanoelectrospray ionization tandem mass spectrometry (MS), the peptides obtained were desalted and concentrated on POROS R2 reverse-phase material (Applied Biosystems, Foster City, CA). The peptides were then eluted with 60% acetonitrile in 5% formic acid directly into a nanoelectrospray capillary needle. Mass spectra were acquired on a QSTAR Pulsar i quadrupole time-of-flight tandem mass spectrometer (Applied Biosystems/MDS-Scics, Toronto, Ontario, Canada) equipped with a nanoelectrospray ion source (Proxeon, Odense, Denmark) as described previously (42). Fragmentation by tandem MS yielded a stretch of amino acid sequence, together with its location in the peptide (sequence tag). With this sequence tag information, appropriate protein databases were searched using Mascot search software (Matrix Science, London, United Kingdom). These searches were not successful because the corresponding protein from M. ulcerans was not in the database. Therefore, the amino acid sequences of the peptides were determined (de novo tandem MS sequencing) to perform database searches based on sequence homology using FASTA software (Genetics Computer Group, Madison, WI).

Sequence analysis of the M. ulcerans 18-kDa shsp encoding gene. For sequence analysis, the 18-kDa shsp encoding gene was amplified by PCR using the primers 5′-CCATTCGTGATGCGTACCGACCCG-3′ and 5′-CTCGAGGACCTTATC ACCTCGG-3′. DNA was extracted as described previously (37), and amplifications were performed using the following profile: 5 min at 96°C; followed by 30 cycles of 1 min at 96°C, 1 min at 63°C, and 1 min at 72°C; and finally by 7 min at 72°C. Amplicons were purified by using a PCR product purification kit (QIAagen) and then sequenced by using an ABI Prism 310
genetic analyzer (Perkin-Elmer). All sequences were reconfirmed at least twice using independent PCR products.

Recombinant expression of the *M. ulcerans* 18-kDa shsp. The *M. ulcerans* 18-kDa shsp was recombinantly expressed in *Escherichia coli* as His-tagged fusion protein (M1V2M3—A149LEH6), comprising the entire open reading frame of 149 codons with one amino acid exchange (L2 to V2). Briefly, the 453-bp PCR product generated from genomic DNA of the Ghanaian *M. ulcerans* isolate ITM 97-483, using the primers described above, was digested with the restriction enzymes NcoI and XhoI and cloned into the pETBLUE2 vector using its NcoI and XhoI sites. Competent *E. coli* Tuner cells (pLac; Novagen) were transformed and expression of the fusion protein was induced by the addition of 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside; Calbiochem) for 4 h at 37°C. Cells

![Graph A](image1.png)

**FIG. 2.** Analysis of the in-gel-digested band with an apparent molecular mass of 18 kDa. (A) Survey mass spectrum of the tryptic in-gel digest. The arrows indicate the doubly charged ion signals of the three peptides, which were sequenced de novo by tandem MS. (B) Tandem mass spectrum of the [M+2H]2+ precursor ion (m/z = 576.32) of peptide M1 (mass determined = 1,150.64 Da). The database search was performed with the singly charged fragment ions labeled y1 to y10.

![Graph B](image2.png)
with a broad range of blot analysis, the generated antisera exhibited cross-reactivity with all 21 mycobacterial species tested, others were more specific with the antigens not present in these two mycobacterial pathogens, B-cell hybridomas were generated with spleen cells of the immunized mice. Although some of the obtained MAbs showed cross-reactivity with different geographical origins tested (data not shown).

Identification of the MAb DD2.5/2.6/3.6-reactive protein. For the identification of the MAbs DD2.5/2.6/3.6-reactive 18-kDa protein, an *M. ulcerans* whole-cell lysate was fractionated by reversed-phase high-pressure liquid chromatography. Fractions in which the immune reactive protein was enriched were identified by Western blot analysis, the peak fractions were pooled, and the 18-kDa band was cut out from an SDS-PAGE gel loaded with the pooled material. The excised protein was analyzed by matrix-assisted laser desorption ionization MS (Fig. 2). The three peptide sequences obtained matched sequence stretches (Table 1) of an *M. leprae* protein (Swiss-Prot: 18kd_mycle) designated 18-kDa shsp (HSP 16.5) (5). An *M. ulcerans* homologue with 79% protein identity (Fig. 3) and 85% identity at the DNA sequence level was identified by homology search in the *M. ulcerans* genome project database (genopole.pasteur.fr/Mycle/BuruliList.html). Codon analysis of the *M. ulcerans* gene predicted a functional open reading frame of 149 amino acids and a molecular mass of 16,556 Da. A gene bank BLAST search identified homologues of 18-kDa shsp in *M. intracellulare*, *M. avium*, and *M. leprae* with protein sequence identities of 79.5, 71.5, and 78.8%, respectively, and extensive interspecies diversity focused on the carboxyl terminus (Fig. 3). In contrast, no homologues are present in the *M. tuberculosis* and *M. bovis* genomes.

To confirm the identification of the 18-kDa shsp as a target for MAbs DD2.5, DD2.6, and DD3.6, the complete coding sequence of the *M. ulcerans* homologue was expressed as a carboxy-terminally hexahistidine-tagged fusion protein in *E. coli*. All three MAbs showed reactivity with the affinity-purified recombinant protein in Western blot analysis (data not shown).

Interspecies immunological cross-reactivity of the 18-kDa shsp was analyzed further by Western blot analysis with mouse antisera raised against the recombinant *M. ulcerans* 18-kDa shsp. Like the three MAbs DD2.5, DD2.6, and DD3.6, all six antisera tested exhibited cross-reactivity with *M. chelonae* strains.

### RESULTS

Interspecies cross-reactivity patterns of MAbs raised against a whole-cell lysate of *M. ulcerans*. To identify immunodominant antigens of *M. ulcerans*, mice were immunized with a complete lysate of the Ghanaian *M. ulcerans* strain 97-610. In Western blot analysis, the generated antisera exhibited cross-reactivity with a broad range of *M. bovis* and *M. tuberculosis* antigens (data not shown). In order to identify *M. ulcerans* antigens not present in these two mycobacterial pathogens, B-cell hybridomas were generated with spleen cells of the immunized mice. Although some of the obtained MAbs showed cross-reactivity with all 21 mycobacterial species tested, others were more specific (Fig. 1). The highest selectivity was observed with three MAbs, designated DD2.5, DD2.6, and DD3.6, which stained an *M. ulcerans* protein with an apparent molecular mass of 18 kDa. Although generated from two different mice, all three MAbs shared the same limited interspecies cross-reactivity pattern, i.e., they only reacted with *M. chelonae*, where a protein with an apparent mass of 20-kDa was recognized. In indirect immunofluorescence assays only *M. ulcerans* was stained by MAbs DD2.5, DD2.6, and DD3.6 (data not shown), indicating that cross-reactivity with *M. chelonae* was too low in affinity to be detectable in this assay. The MAbs stained an 18-kDa band in all 10 *M. ulcerans* isolates of different geographical origins tested (data not shown).

Identification of the MAb DD2.5/2.6/3.6-reactive protein. For the identification of the MAbs DD2.5/2.6/3.6-reactive 18-kDa protein, an *M. ulcerans* whole-cell lysate was fractionated by reversed-phase high-pressure liquid chromatography. Fractions in which the immune reactive protein was enriched were identified by Western blot analysis, the peak fractions were pooled, and the 18-kDa band was cut out from an SDS-PAGE gel loaded with the pooled material. The excised protein was analyzed by matrix-assisted laser desorption ionization MS (Fig. 2). The three peptide sequences obtained matched sequence stretches (Table 1) of an *M. leprae* protein (Swiss-Prot: 18kd_mycle) designated 18-kDa shsp (HSP 16.5) (5). An *M. ulcerans* homologue with 79% protein identity (Fig. 3) and 85% identity at the DNA sequence level was identified by homology search in the *M. ulcerans* genome project database (genopole.pasteur.fr/Mycle/BuruliList.html). Codon analysis of the *M. ulcerans* gene predicted a functional open reading frame of 149 amino acids and a molecular mass of 16,556 Da. A gene bank BLAST search identified homologues of 18-kDa shsp in *M. intracellulare*, *M. avium*, and *M. leprae* with protein sequence identities of 79.5, 71.5, and 78.8%, respectively, and extensive interspecies diversity focused on the carboxyl terminus (Fig. 3). In contrast, no homologues are present in the *M. tuberculosis* and *M. bovis* genomes.

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### TABLE 1. Amino acid sequences of the *M. ulcerans* 18-kDa protein-derived peptides determined by de novo tandem MS sequencing

| Peptide [M + H]^+ | Mass (Da) | *M. ulcerans* sequence determined by tandem MS | Corresponding *M. leprae* 18-kDa shsp sequence |
|-------------------|-----------|-----------------------------------------------|-----------------------------------------|
| M₁                | 1,151.64  | IAASYTENGKL | ILASYOEGVLK                           |                           |
| M₂                | 1,177.66  | FQAVQVLTASR | FAEOVQVLTASR                           |                           |
| M₃                | 1,328.75  | QVLGVEHDLTAR | QVLGVEHDLTAR                          |                           |

*Leu and Ile cannot be discriminated by the technology applied and are therefore interchangeable. Amino acid differences are indicated in boldface.*

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FIG. 3. Comparison of the predicted amino acid sequences of the 18-kDa shsp of different mycobacterial species. Identity of the *M. ulcerans* reference sequence is indicated by dashes (-), deletions are indicated by a dot (.) . Gray boxes indicate the positions of peptides identified by matrix-assisted laser desorption ionization–time of flight tandem MS. The GenBank accession numbers were as follows: *M. leprae*, AL583923; *M. intracellulare*, L12240; and *M. avium*, AE017238.
(Fig. 4). In addition, antisera cross-reacted with an 18-kDa *M. leprae* protein, which was only stained by MAbs DD2.5, DD2.6, and DD3.6 when they were used at very high (≥5 μg/ml) concentrations (data not shown). When a set of 10 overlapping synthetic 20mer peptides spanning the entire sequence of the *M. ulcerans* 18-kDa shsp was tested by enzyme-linked immunosorbent assay, MAbs DD2.5, DD2.6, and DD3.6 showed no reactivity, and only some of the antisera bound weakly to the C-terminal peptide.

**Sequence conservation, subcellular localization, and expression of the *M. ulcerans* 18-kDa shsp in Buruli ulcer lesions.** Only four single-nucleotide polymorphisms were detected when the 18-kDa shsp encoding genes of 10 *M. ulcerans* isolates of diverse geographical origin were compared by PCR amplification and DNA sequence analysis of PCR products (Fig. 3). Two single-nucleotide polymorphisms, one nonsynonymous (G/T424) and one synonymous (T/C374), distinguished the sequence of strain 7922 from French Guiana from that of the Ghanaian genome project reference strain Agy-99. Strain 8756 from Japan exhibited two synonymous single-nucleotide polymorphisms (G/A278 and C/T395) with respect to the reference sequence. Sequences from the other eight *M. ulcerans* strains analyzed were identical with the Agy-99 reference sequence.

The MAb DD2.5/6/3.6-reactive band was primarily found in *M. ulcerans* cell wall fractions (Fig. 5A) but was undetectable in cytosol fractions (Fig. 5A) and in culture filtrate (not shown). The *M. ulcerans* 18-kDa shsp was detectable in human tissue lysates from the center of excised Buruli ulcer lesions (Fig. 5B). No staining was observed in lysates from the healthy margins of the excised tissue (Fig. 5B).

**Reactivity of human sera with the recombinant 18-kDa shsp of *M. ulcerans*.** In Western blot analyses, the majority of sera from preulcerative (Fig. 6A), early ulcerative (Fig. 6B), and late-ulcerative (Fig. 6C) Buruli ulcer patients showed reactivity with the recombinant 18-kDa protein of *M. ulcerans*. Whereas 75% (24 of 32) of the patient sera were tested positive, 38% (9 of 24) of the sera from household contacts also showed reactivity (Fig. 6D). Samples from Europeans (Fig. 6F) and from the vast majority of Africans living in regions where Buruli ulcer is not endemic (Fig. 6E) were negative.

**DISCUSSION**

At present it is not clear what proportion of a population living in an African Buruli ulcer focus area is exposed to *M. ulcerans*. In the case of *M. tuberculosis*, an infection remains latent in 90 to 95% of individuals, and progressive disease development is only observed in a minority of infected individuals. Likewise, a significant proportion of individuals infected by *M. ulcerans* may not develop the disease. Few data are available on coinfections of human immunodeficiency virus (HIV) and *M. ulcerans*, and it is not entirely clear whether HIV infection is a risk factor for Buruli ulcer (1). Anecdotal evidence indicates that HIV infection affects the outcome of Buruli ulcer disease (21).

For the assessment of the prevalence of exposure, a test is required that is negative for nonexposed persons from regions where the disease is not endemic and positive for a significant proportion of exposed individuals in region where it is endemic. The test should discriminate between immune responses against *M. ulcerans* and other mycobacteria, in particular *M. tuberculosis* and *M. bovis* BCG. Serological studies performed with complex antigen preparations have suggested that serological tests may be useful in the diagnosis and surveillance of Buruli ulcer (9, 16, 31). In view of the presence of species cross-reactive antibodies in sera of Africans living in regions where Buruli ulcer is endemic, thoroughly selected recombinantly expressed target antigens are required. Our search for immunodominant proteins of *M. ulcerans* has identified the 18-kDa shsp as promising candidate for a serological test suitable to monitor the exposure of a population to *M. ulcerans*. While the 18-kDa shsp has no homologue in the...
genomes of *M. bovis* and *M. tuberculosis*, homologues have been described in *M. leprae*, *M. marinum*, *M. intracellulare*, and *M. avium*. Mouse sera raised against the recombinantly expressed *M. ulcerans* protein showed cross-reactivity with a 18-kDa protein of *M. leprae* and a 20-kDa protein of *M. chelonae* but not with *M. intracellulare* and *M. avium* lysates. The 18-kDa shsp of *M. leprae* has been evaluated as target antigen for serological and cellular diagnostic tests for leprosy (10, 29, 43). Whereas the *M. leprae* protein seems to share epitopes with an unidentified *M. tuberculosis* antigen (30, 36, 41), our MAbs and mouse sera specific for the *M. ulcerans* 18-kDa shsp did not cross-react with *M. tuberculosis* and *M. bovis* BCG lysates. Furthermore, sera from individuals living in regions where Buruli ulcer is not endemic were largely negative. These results indicate that immune responses against environmental mycobacteria, such as *M. chelonae*, which is widely distributed in the environment (4, 22) and expresses a cross-reactive homologue of the 18-kDa shsp, do not obscure the results with the *M. ulcerans* 18-kDa shsp-based serological test.

Like its homologue in *M. leprae* (23), the 18-kDa shsp of *M. ulcerans* is associated with the cell wall fraction. It has been postulated that the 18-kDa shsp of *M. leprae* is relevant for the survival of the mycobacteria within macrophages (7). While *M. ulcerans* is largely an extracellular pathogen, it appears to be captured by phagocytes and transported to draining lymph nodes within host cells during the early stage of infection (6). The *M. ulcerans* 18-kDa shsp may play a role in this early intracellular stage of the infection and protect the mycobacteria in extreme environmental conditions by stabilizing the cell wall (25, 26).

Although it is clear from many epidemiological studies that proximity to wetlands is a risk factor for *M. ulcerans* infection, the exact mode of transmission is not clear (20). Recent field and laboratory studies have implicated aquatic insects in the transmission of the pathogen (25, 33), and it has been demonstrated that mycolactone toxin-producing *M. ulcerans* isolates are able to invade the salivary glands of water insects (24). *M. ulcerans* DNA has also been detected by PCR in aquatic snails, fish, and the biofilm of aquatic plants (27, 38, 40), but the contributions of these elements of the environment in transmission has remained largely unknown. *M. ulcerans* may often reach the human dermis through minor wounds or skin abrasions.
sions via contact with \(M. ulcers\)-containing environmental reservoirs (28). Our analysis of sera from healthy household contacts indicates that exposure to \(M. ulcers\) leads only in a minority of exposed individuals to clinical disease. Most of the others may only develop transient infection foci, and even nodular lesions may resolve spontaneously (35). Immune responses in healthy household contacts have also been described in an Australian study (16), where a lower background staining than with African sera facilitated analysis with cellular extracts. Our preliminary analysis of sera from Africans living in regions where Buruli ulcer is endemic indicate that exposure is common in these environments also among nonhousehold contacts. Future prospective analysis of cellular and humoral immune responses with recombinant \(M. ulcers\) proteins in a population living in a region of Africa where this organism is highly endemic should give better insight into patterns of exposure. Such studies may also lead to the identification of surrogate markers of protection crucial for the development of a vaccine against \(M. ulcers\) infection.

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