Infection with *Mycobacterium ulcerans* Induces Persistent Inflammatory Responses in Mice

Martinha S. Oliveira,1† Alexandra G. Fraga,1† Egídio Torrado,1 António G. Castro,1 João P. Pereira,1 Adhemar Longatto Filho,1,2 Fernanda Milanezi,1,3 Fernando C. Schmitt,1,3 Wayne M. Meyers,4 Françoise Portaels,5 Manuel T. Silva,1,6 and Jorge Pedrosa1,*

Life and Health Sciences Research Institute, School of Health Sciences (ICVS), University of Minho, Braga, Portugal; Division of Pathology of Adolfo Lutz Institute, São Paulo, Brazil; Institute of Molecular Pathology and Immunology, University of Porto, Porto, Portugal; Armed Forces Institute of Pathology, Washington, D.C.; Mycobacteriology Unit, Department of Microbiology, Institute of Tropical Medicine, Antwerp, Belgium; and Institute for Molecular and Cell Biology, Porto, Portugal

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Buruli ulcer (BU) is a devastating, necrotizing, tropical skin disease caused by infections with *Mycobacterium ulcerans*. In contrast to other mycobacterioses, BU has been associated with minimal or absent inflammation. However, here we show that in the mouse *M. ulcerans* induces persistent inflammatory responses with virulence-dependent patterns. Mycolactone-positive, cytotoxic strains are virulent for mice and multiply progressively, inducing both early and persistent acute inflammatory responses. The cytotoxicity of these strains leads to progressive destruction of the inflammatory infiltrates by postapoptotic secondary necrosis, generating necrotic acellular areas with extracellular bacilli released by the lysis of infected phagocytes. The necrotic areas, always surrounded by acute inflammatory infiltrates, expand through the progressive invasion of healthy tissues around the initial necrotic lesions by bacteria and by newly recruited acute inflammatory cells. Our observations show that the lack of inflammatory infiltrates in the extensive areas of necrosis seen in advanced infections results from the destruction of continuously produced inflammatory infiltrates and not from *M. ulcerans*-induced local or systemic immunosuppression. Whether this is the mechanism behind the predominance of minimal or absent inflammatory responses in BU biopsies remains to be elucidated.

Pathogenic mycobacteria are intracellular parasites that are responsible for several clinically important infections in humans and animals. The most frequent mycobacterial infections in humans are caused by *Mycobacterium tuberculosis* and *M. leprae*. However, a unique group of mycobacteria has been emerging and comprises infections caused by *M. marinum*, *M. hemophilum*, and *M. ulcerans* (12). These are slow-growing mycobacteria with some genetic relatedness (71) and common peculiar characteristics. These mycobacteria have optimal growth temperatures of 28 to 33°C and infect primarily the cooler parts of the body, mainly the skin. They have cytotoxic activity (17, 56, 57) and, as a consequence, produce necrotizing lesions (3, 7, 12, 72).

Buruli ulcer (BU), caused by *M. ulcerans*, has become the third most prevalent mycobacteriosis throughout the world, after tuberculosis and leprosy, with higher incidence in some African regions (11, 74). This view gained a consensus status over time to a chronic, mononuclear, granulomatous pattern (10, 36). The histopathology of lesions produced by *M. tuberculosis* and *M. marinum* shows these typical features of mycobacterial infections (3, 7, 12, 44, 72). It is therefore intriguing that *M. ulcerans*, despite being related to those mycobacteria, would produce a disease where minimal or absent inflammatory responses have been predominantly observed (6, 8, 9, 27, 30, 31). This view gained a consensus status and became a hallmark of the histopathology of *M. ulcerans* infection in human disease (1, 12, 15, 21–25, 35, 45, 48, 52, 64). Experimental *M. ulcerans* infections in mice, rats, guinea pigs, and nine-banded armadillos led to contradictory observations regarding induction of inflammatory responses. In some stud-
ies, the occurrence of an inflammatory response was reported (38, 40, 57), but other studies showed minimal inflammation associated with M. ulcerans infection (22, 23, 75). Moreover, the dynamics of the infection process and host inflammatory response at different stages of infection with M. ulcerans are still poorly understood.

The present study was undertaken to evaluate the different stages of experimental M. ulcerans infection and associated cellular inflammatory responses. We examined the cytotoxicity and pathogenicity for mice of different clinical isolates of M. ulcerans by evaluating cell death of infected macrophages in vitro and lesions produced in a footpad mouse model of infection. We analyzed M. ulcerans-induced disease from the early stage of infection until later stages associated with ulceration, which allowed us to characterize the dynamics of the infectious process. We found that the mycolactone-producing strains have cytotoxic activity and proliferate in mouse footpads, inducing pathology, while the mycolactone-negative strain is nontoxic and nonvirulent. We also found that M. ulcerans consistently induces an early, acute neutrophilic inflammatory response that is independent of virulence, followed by prolonged cellular responses that differ according to the strain. With strains virulent for mice, the acute inflammatory infiltrate persisted throughout infection and consisted primarily of neutrophils and some macrophages and lymphocytes surrounding a central, expanding, necrotic acellular focus. In contrast, with the avirulent strain, the initial acute inflammatory response switched to a chronic inflammatory response with a persistent mononuclear infiltrate with few neutrophils, associated with granuloma-like structures and the absence of necrosis.

**MATERIALS AND METHODS**

**Mycobacterial strains and preparation of inocula.** The M. ulcerans strains used in this study were selected based on our preliminary studies that showed different degrees of virulence in mice. Moreover, the genetic and phenotypic characteristics of these strains, including production of mycolactone, had been previously described (4, 5, 45, 67). The strains are from the collection of the Institute of Tropical Medicine (ITM), Antwerp, Belgium, where they have been kept freeze-dried at −80°C before being passaged to Lowenstein-Jensen (LJ) medium for the present study, as well as for recently published studies on their ability to produce mycolactone (45) and, in the case of strains 5114 and 98-912, on the structure of the plasmid that carries genes responsible for mycolactone synthesis (68). Strain 5114 was isolated from a case of ulcerative BU in Mexico and was found to produce mycolactones (45) due to the loss of key genes involved in mycolactone synthesis (68). Strain 5114 was recovered from slants of LJ medium, diluted in phosphate-buffered saline (PBS) to a final concentration of 1 mg/ml, and vortexed vigorously with 2-mm glass beads.

**Culture of murine bone-marrow derived macrophages.** Macrophages were derived from the bone marrow as follows. Mice were euthanized with CO2 and femurs removed under aseptic conditions. Bones were flushed with 5 ml cold Hanks’ balanced salt solution (HBSS) (Gibco, Paisley, United Kingdom). The resulting cell suspension was centrifuged at 500 × g and resuspended in Dulbecco’s modified Eagle’s medium (DMEM) (Gibco) supplemented with 10 mM HEPES (Sigma, St. Louis, MO), 1 mM sodium pyruvate (Gibco), 10 mM glucose (Gibco), 10% heat-inactivated fetal bovine serum (Sigma), and 10% L929 cell conditioned medium (complete DMEM [cDMEM]). To remove fibroblasts or differentiated macrophages, cells were cultured for a period of 4 hours on cell culture dishes (Nunc, Naperville, IL) with cDMEM. Nonadherent cells were collected with warm HBSS, centrifuged at 500 × g, distributed in 24-well plates at a density of 5 × 10⁴ cells/well, and incubated at 37°C in a 5% CO2 atmosphere. A 0.1-ml amount of L929 cell conditioned medium was added 4 days after seeding, and medium was renewed on the seventh day. After 10 days in culture, cells were completely differentiated into macrophages. Twelve hours before infection, macrophages were incubated at 32°C in a 5% CO2 atmosphere and maintained until the end of the experimental infection as described elsewhere (50).

**Footpad model of infection.** Mice were infected in the left hind footpad with 0.03 ml of M. ulcerans suspensions containing the numbers of AFB indicated for each experiment. The right hind footpad was used as a control. At different time points after infection, four mice per group were sacrificed, and peritoneal cells were recovered by peritoneal lavage using 4 ml of PBS. Total leukocyte numbers were determined, and differential leukocyte counts were performed by cytopsin preparations fixed with 10% formalin in ethanol and stained with Hemacolor (45). Tissue samples were fixed in 10% phosphate-buffered formalin. The whole paws were decalcified using the method of Shepard and McRae (61). Sections (6 μm) were processed for light microscopy. Digital images were captured by using a Axioscam HRc camera (Zeiss, Hallebergmoos, Germany). As a control for cytotoxic activity of M. ulcerans strains, uninfected BMDM were analyzed at the same time intervals.

**Histological studies.** All excised tissues from control and infected footpads were fixed in 10% phosphate-buffered formalin. The whole paws were decalcified (Thermo Shandon TBO-1, Runcorn, United Kingdom) and embedded in paraffin. Longitudinal sections of the paws (4 μm) were processed for light microscopic studies after hematoxylin-eosin (HE) or ZN staining. For each sample, serial longitudinal sections of the entire paw were viewed under the microscope to ensure that representative areas of the lesions were analyzed. Several animals were studied for each time point as indicated in Table 1, and all histological...
observations were carried out blind. For TUNEL staining of paraffin sections, the In Situ Cell Death Detection Kit, AP (Roche Diagnostics), was used according to the manufacturer’s instructions with the final contrast stain omitted. TUNEL-positive nuclei stain red; normal nuclei are unstained.

**Statistical analysis.** Statistical significance of values was determined using the Student t test.

## RESULTS

We first characterized the in vitro cytotoxicity as well as the in vivo pathology induced by three selected strains of *M. ulcerans*. Next, we analyzed the cellular inflammatory responses induced by these strains in the mouse peritoneal cavity and footpads.

*M. ulcerans* clinical isolates show different cytotoxicities against BMDM. The genetic heterogeneity within *M. ulcerans* clinical isolates, which is caused by several factors, including instability and mutability of genes encoding the machinery for the synthesis of mycolactone, results in variable degrees of toxigenicity (68, 69). Cell lines treated with *M. ulcerans* culture filtrates, acetone-soluble lipids containing mycolactone, or purified mycolactone exhibit typical cytopathic changes (21–23, 45). However, the cytotoxicity of live *M. ulcerans* bacilli from different clinical isolates on infected macrophages has not been characterized. We first established an in vitro model of BMDM infection at 32°C in order to evaluate the cytotoxic activities of the different clinical isolates of *M. ulcerans* used in this study. Macrophage cultures were infected with *M. ulcerans* strain 5114, 97-1116, or 98-912 at an MOI of 1:1. No significant alterations were found in monolayers infected with strain 5114 compared to uninfected BMDM at 4 days (Fig. 1A and B) and at 8 days (data not shown) after infection. In contrast, strains 98-912 and 97-1116 are cytotoxic for macrophages, as assessed by cell rounding, shrinkage, and detachment of more than 90% of macrophages at 4 days (Fig. 1C and D). Positive TUNEL staining in a high proportion of macrophages (Fig. 1E) points to the apoptotic nature of that cytotoxicity.

### TABLE 1. Assessment of histopathological features after infection with different strains of *M. ulcerans*

<table>
<thead>
<tr>
<th>Histopathological parameter</th>
<th><em>M. ulcerans</em> strain</th>
<th>No. of positive cases/total studied cases on postinfection day:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acute inflammation (predominant neutrophilic infiltrate)</td>
<td>98–912 5114</td>
<td>2 7 15 24 43 60 <em>a</em></td>
</tr>
<tr>
<td>Chronic inflammation (predominant macrophagic-lymphocytic infiltrate)</td>
<td>98–912 5114</td>
<td>0 3 6 6 5 2 2 <em>b</em></td>
</tr>
<tr>
<td>Presence of granuloma-like structures</td>
<td>98–912 5114</td>
<td>0 3 6 6 5 2 2 <em>b</em></td>
</tr>
<tr>
<td>Leukocyte destruction (apoptosis/necrosis)</td>
<td>98–912 5114</td>
<td>0 3 6 6 5 2 2 <em>b</em></td>
</tr>
<tr>
<td>Subcutaneous tissue necrosis</td>
<td>98–912 5114</td>
<td>0 3 6 6 5 2 2 <em>b</em></td>
</tr>
</tbody>
</table>

*—*, animals were sacrificed after emergence of ulceration.

**FIG. 1.** Cytotoxicity of *M. ulcerans* clinical isolates to BMDM. BMDM were not infected (A) or were infected with *M. ulcerans* strain 5114 (B), 97-1116 (C), or 98-912 (D and E) at an MOI of 1:1. Macrophages were photographed 4 days after infection by phase-contrast microscopy (A to D) or with fluorescence microscopy for samples stained with TUNEL (E). Positive TUNEL staining (yellow-green fluorescent nuclei in E) points to the apoptotic nature of cell death induced by the cytotoxic strain 98–912. Bars, 50 μm.
Since (i) the mycolactone-negative strain 5114 is noncytotoxic for macrophages, whereas the mycolactone-positive strains 98-912 and 97-1116 are cytotoxic, and (ii) this cytotoxicity is associated with cytopathic changes similar to those induced in fibroblast and macrophage cell lines by purified mycolactone (22, 23), we conclude that cytotoxicity of strains 98-912 and 97-1116 for BMDM correlates with mycolactone production.

Cytotoxic M. ulcerans isolates are virulent for mice. We measured over time the mean swelling of footpads infected with different strains of M. ulcerans as a macroscopic parameter for monitoring pathology. Significant differences were found in the mean swelling after inoculation of 5 log10 AFB of each M. ulcerans strain in the mouse footpad. Swelling was evident in mice infected with the cytotoxic M. ulcerans strain 98-912 during the second week of infection (Fig. 2A and B), and ulceration occurred by the end of the fourth week. Strain 97-1116 induced swelling of the footpad during the fourth week (Fig. 2A) and ulceration during the seventh week. In contrast, the noncytotoxic M. ulcerans strain 5114 was found not to induce significant swelling of the footpad up to 90 days of infection (Fig. 2A and B) or at 12 months postinoculation (data not shown). Infection of mice with 2 log10 AFB of M. ulcerans 98-912 showed that this strain still induced footpad swelling, albeit delayed, at concentrations 1,000-fold lower than the inoculum of M. ulcerans 5114 that did not induce measurable swelling (Fig. 2B).

To assess if the severity of lesions induced by M. ulcerans strains was associated with their capacity to proliferate in the host, we performed AFB counts in footpad homogenates. We found that M. ulcerans strains proliferated differently over the experimental period of infection. AFB counts following infection by M. ulcerans 98-912 increased significantly ($P < 0.001$) from 5.0 log10 to 7.90 log10 between days 0 and 26 of infection (Fig. 2C). In mice infected with strain 97-1116, AFB counts increased approximately 2 log10 ($P < 0.001$) from day 0 to day 39 postinfection (Fig. 2C). Conversely, M. ulcerans strain 5114 increased only from 5.06 log10 to 5.69 log10 ($P < 0.01$) between days 0 and 16 postinfection and reached a plateau after 3 months (Fig. 2C) that remained stable after 12 months of infection (data not shown).

These results show that mycolactone-positive, cytotoxic strains of M. ulcerans are virulent for mice, while the mycolactone-negative, noncytotoxic strain is avirulent.

M. ulcerans induces an early acute inflammatory response in the peritoneal cavity. Mycobacteria, such as those caused by the M. ulcerans-related species M. tuberculosis, M. marinum, and M. hemophilum, have been consistently associated with acute and chronic recruitment of inflammatory cells to the site of infection (3, 7, 10, 36, 44, 51, 59, 60, 62, 72). However, reports on the histopathology of M. ulcerans infection are intriguing, since several authors describe minimal or absent cellular inflammation.

The mouse peritoneal model of infection has been used to characterize the inflammatory cellular response to mycobacteria because it allows a precise qualitative and quantitative analysis of leukocyte populations (2, 51, 62). We used this model to evaluate quantitatively the early inflammatory response to M. ulcerans inoculation. After intraperitoneal infection with 6 log10 M. ulcerans 5114 AFB, 97-1116, or 98-912,

FIG. 2. Footpad swelling and proliferation of different strains of M. ulcerans. Panels A and C show swelling and AFB counts, respectively, of BALB/c mice footpads infected subcutaneously with 5.0 log10 AFB of M. ulcerans 98-912 (■), 5.2 log10 AFB of M. ulcerans 97-1116 (▲), or 5.1 log10 AFB of M. ulcerans 5114 (●). Panel B shows swelling of footpads infected with 5.5 log10 AFB (●) or 2.5 log10 AFB (□) of M. ulcerans 98-912 or 5.5 log10 AFB of M. ulcerans 5114 (●). Footpad swelling was measured in eight mice from each group. Significant differences in footpad swelling were calculated by comparing infected and noninfected mice. Significant differences in AFB counts were determined by comparing the bacterial loads of five footpads from each group with the respective initial inoculum. Calculations were performed using Student’s t test (*, $P < 0.05$; **, $P < 0.01$; ***; $P < 0.001$). Mice were sacrificed for ethical reasons after the emergence of ulceration (†). Results are from one representative of three independent experiments. Error bars indicate standard deviations.
peritoneal exudates were collected at different time points. Total cell counts and differential leukocyte quantification were performed over a 2-week period. We found a significant recruitment of neutrophils and macrophages in mice infected with strains 5114 and 98-912 (Fig. 3) and 97-1116 (data not shown). The number of neutrophils increased more than 50-fold ($P < 0.001$), peaked at 8 h postinfection, and declined afterwards but remained 10-fold above the initial value (Fig. 3). The number of monocytes/macrophages increased significantly 7 days after inoculation ($P < 0.05$) (Fig. 3). No significant differences in other leukocyte populations were detected. Control mice injected intraperitoneally with PBS did not show a significant influx of neutrophils or macrophages (data not shown).

These results show for the first time that early recruitment of high numbers of neutrophils followed by an influx of monocytes/macrophages is a feature of $M. ulcersans$ infection in the mouse, irrespective of the virulence of the isolates.

$M. ulcersans$ induces extensive inflammatory responses in the mouse footpad. Experimental infections of the mouse tail, ear, or footpad present several advantages over the peritoneal model for studying long-term infections by $M. ulcersans$, not only because of the nonpermissive temperature of the peritoneal cavity but also because it parallels the BU disease that only because of the nonpermissive temperature of the peritoneal cavity but also because it parallels the BU disease that otherwise only occurs in the intracutaneous model with a high inoculum. Therefore, we used the footpad model of infection to characterize different phases of the inflammatory response to infection with $M. ulcersans$.

We performed a semiquantitative blind assessment of several histopathological parameters in sections of hind footpad tissue infected with $5 \log_{10}$ AFB of $M. ulcersans$ 98-912, 97-1116, or 5114, as summarized in Table 1. We found that the three strains induced an early and intense acute inflammatory cellular response in infected footpads. Within the first hours after inoculation, we observed acute inflammatory responses showing a predominance of neutrophils with scattered macrophages and lymphocytes (Fig. 4, A, B, G, H, M, and N), and with numerous bacilli within neutrophils and macrophages (not shown), as previously described (16).

However, the progression of histological features associated with pathology during experimental infection is largely dependent on the virulence of the $M. ulcersans$ strain. Leukocyte destruction became apparent in the initial acute inflammatory infiltrate as early as 24 h after inoculation of the mouse-virulent strains 98-912 and 97-1116, as described in more detail in the next section. This cell death process leads to the production of necrotic, acellular foci (Fig. 4, E, F, K, and L) that continuously expand during infection. The centrifugal advancement of cell destruction leaves behind cell debris and freed extracellular bacilli that accumulate in the necrotic areas (Fig. 5A, D, F, and G). This is accompanied by progressive invasion of healthy tissues by acute inflammatory infiltrates around the areas where previous infection led to necrotic destruction of acute infiltrates (Fig. 4E and K and 5A and D). The initiation of a switch from acute infiltrates, predominantly neutrophilic, to infiltrates with a majority of mononuclear cells, characteristic of chronic inflammation, as seen in some mice at day 7 postinfection, is not fully accomplished (Table 1).

In contrast, mice infected with the avirulent strain 5114 showed absence of necrotic alterations during the whole experimental period (Fig. 4M to R). The initial neutrophilic inflammatory process (Fig. 4M and N) was gradually replaced...
FIG. 4. Histological sections of footpads of mice infected with *M. ulcerans*. BALB/c mice were infected with 5.9 log₁₀ AFB of *M. ulcerans* 98-912 (A to F), 5.5 log₁₀ AFB of *M. ulcerans* 97-1116 (G to L), or 5.8 log₁₀ AFB of *M. ulcerans* 5114 (M to R). Footpads were collected at the indicated times postinfection, and tissue sections were stained with HE (A, B, C, E, G, I, K, M, N, O, and Q) or ZN (D, F, J, L, P, and R). Magnifications, ×10 (A, C, E, G, I, K, M, O, and Q) and ×350 (B, D, F, H, J, L, N, P, and R). Panels A, B, G, H, M, and N show an acute inflammatory response with predominance of neutrophils (arrows) early after infection by all strains of *M. ulcerans*. Panels C to F and I to L depict advanced stages of infection with *M. ulcerans* 98-912 or 97-1116, respectively, and show necrosis of the central focus of infection (E, F, K, and L, asterisks) and high numbers of extracellular bacteria (F and L), surrounded by a band of acute cellular infiltrate (E and K, arrowheads). In contrast, advanced stages of infection with *M. ulcerans* 5114 show predominantly mononuclear infiltrates with intracellular bacilli, epithelioid transformation (O to R), and granuloma-like organization (P). Results are from one representative experiment of two independent experiments.
at the second week after infection by a predominantly lymphocytic and macrophagic infiltrate (Fig. 4O to R) with epithelioid transformation, in the presence of a small number of neutrophils (Table 1). During the remaining time of experimental infection, a predominance of lymphocytes, macrophages, and giant cells was maintained, together with granuloma-like organization (Fig. 4P) and some neutrophils. We found intracellular bacilli throughout the whole experimental period of infection (Fig. 4P and R).

These observations confirmed the occurrence of an acute cellular inflammation in the subcutaneous tissue, with the typical early and extensive recruitment of neutrophils in response to the infection with different strains of *M. ulcerans*, irrespective of their virulence for mice. In addition, our data showed that areas of cellular inflammation persist until the later stages of infection, with a cellular pattern varying according to the virulence of the strain.

**Mouse-virulent strains of *M. ulcerans* induce postapoptotic secondary necrosis.** It has been shown that *M. ulcerans* and its exotoxin mycolactone induce cell destruction in guinea pig skin (22, 23), in fibroblast and macrophage cell lines (22, 23), and in BMDM (Fig. 1E) by a cell death process with characteristics of apoptosis. We found that the destructive process affecting cell populations of acute inflammatory infiltrates in footpads infected with mouse-virulent *M. ulcerans* strains, described in the previous section, shows morphological features of apoptotic cell death, namely pyknosis and karyorrhexis (77) (Fig. 6A, C, and E) and TUNEL positivity of pyknotic and fragmented nuclei (Fig. 6C). The dying cells were present in areas with bacilli (Fig. 6D and F) or close to bacilli. However, we did not find phagocytosis by macrophages of the cells undergoing apoptosis. This observation, together with the presence of abundant cell debris mixed with cells undergoing apoptosis (Fig. 6B and E), indicates that the cell death process proceeds to secondary necrosis (77). Therefore, massive postapoptotic cell lysis in the areas of acute inflammatory infiltrate rich in or close to bacilli is the process responsible for the production of expanding necrotic, acellular areas.

**DISCUSSION**

In humans, infections by *M. ulcerans* have been extensively characterized by minimal or absent inflammation (1, 6, 8, 9, 12, 15, 21–26, 30, 31, 35, 45, 48, 52, 64), which contrasts with what is known to occur with other mycobacterioses (3, 7, 10, 12, 36, 44, 51, 59, 60, 62, 72). On the other hand, the experimental
models of infection by *M. ulcerans* led to contradictory results regarding inflammatory responses (22, 23, 38, 40, 57, 75). Therefore, comprehensive analyses of infection with different *M. ulcerans* clinical isolates were carried out in mice to understand the dynamics of inflammatory responses in an animal model largely used in the study of experimental infections, including mycobacteriosis (18, 47), and to compare the histopathological features of *M. ulcerans* infections in mice and humans. Here we show that *M. ulcerans* induces relevant, virulence-dependent inflammatory responses in the mouse. The mycolactone-negative, noncytotoxic strain does not produce progressive infection and induces an initial acute neutrophilic response that switches to a chronic mononuclear infiltrate associated with granuloma-like structures, in the absence of necrosis. The two mycolactone-positive, cytotoxic strains multiply progressively and induce an acute inflammatory response that is permanently present during the infectious process, although at spatially restricted areas surrounding a central necrotic, acellular focus that expands with the progress of the disease.

The dynamics of the cellular response against infections has been thoroughly described and is characterized by an early, acute inflammation enriched in neutrophils, followed by recruitment of monocytes that complement the activity of neutrophils (58). This biphasic phagocytic response is characteristic of infections caused by pathogenic *Mycobacterium* species related to *M. ulcerans*, such as *M. tuberculosis* and *M. marinum* (3, 7, 36, 44, 51, 60, 62, 72), and was now found to occur also in the mouse model of *M. ulcerans* infection. We show that the *M. ulcerans*-induced initial inflammatory response is indistinguishable in mice infected with mycolactone-positive or -negative strains. This suggests that production of mycolactone by *M. ulcerans* does not inhibit recruitment of phagocytes. The comprehensive semiquantitative and blind histological analyses carried out in the present study showed that in mice infected with mouse-virulent *M. ulcerans* strains, acute leukocyte responses are present, not only in the beginning of infection but also throughout the entire murine infectious process until ulceration of the footpad. Cell lysis in the acute inflammatory infiltrate became evident early in the infection with these virulent strains and led to the production of expanding necrotic areas. A band of acute cellular infiltrate is constantly present at the periphery of these necrotic areas and is colonized with
intraphagocytic bacilli. In contrast, abundant extracellular bacilli are seen in the expanding necrotic areas devoid of inflammatory cells. These necrotic areas reach considerable extension in advanced lesions and are similar to the necrotic areas predominantly described in human biopsies. Whether the above mechanism is behind the predominance of minimal or absent inflammatory responses in BU biopsies remains to be elucidated.

A switch from the initial acute, predominantly neutrophilic response to a chronic, predominantly mononuclear response, associated with epithelioid and giant cells and formation of granulomas, is typical of mycobacterial infections when a cellular-mediated immunity (CMI) is mounted (36, 44). As shown, in progressive murine infection by virulent M. ulcerans, such a switch is not fully completed. This observation correlates with the finding that progressive infection of the skin, disseminated disease, and osteomyelitis in BU patients were associated with an absence of granulomas in the infected areas (33, 49, 66). Conversely, with the noncytotoxic strain 5114, which is not virulent for mice and does not cause leukocyte death and necrosis of the subcutaneous tissue at any stage of infection, the acute neutrophilic profile of the cellular infiltrate gradually switches to a predominance of mononuclear cells and formation of granuloma-like structures. Not surprisingly, this type of chronic response was observed in BU patients during the healing phase of disease (30, 31, 40, 49, 66, 74).

We were able to observe early and continued acute, predominantly neutrophilic responses to mouse-virulent M. ulcerans strains because the small size of the diseased area allowed the systematic observation of the entire lesion and surrounding healthy tissues even in advanced infections when the areas of necrosis are extensive.

Our interpretation is that the areas of minimal or absent cellular inflammation seen in infected foci in mouse footpads with advanced infections with virulent M. ulcerans strains are due not to the lack of recruitment of inflammatory cells but rather to the destruction of preexistent inflammatory infiltrates. It is likely that such a destruction is due to the potent cytotoxic activity of mycolactone, because lysing leukocytes that colocalize with intracellular bacilli, or are in proximity to extracellular bacilli, show morphological features compatible to those described for the cytopathic activity of mycolactone (22, 23) and do not occur with the mycolactone-negative strain. Whether the observed difference in virulence for mice between strains 98-912 and 97-1116 is related to possible differences in the interaction in murine M. ulcerans infections is the occurrence of similar interactions has not been considered. In fact, BU has been described as a disease with a histopathological pattern characterized by minimal or absent inflammatory responses and by the presence of abundant extracellular bacilli in extensive necrotic, acellular areas (6, 8, 9, 27, 30, 31). Although it is evident that such a histopathological pattern is a diagnostic hallmark of BU, it may not reflect all the aspects of the host-parasite interaction at the active infection foci. Indeed, there are several lines of evidence implicating CMI and delayed-type hypersensitivity responses in human M. ulcerans infections, favoring the possible existence of a phase of intracellularity of the pathogen, as follows. Studies with humans show that resistance to M. ulcerans is associated with the Th1 response to a chronic, predominantly mononuclear response, associated with epithelioid and giant cells and formation of granulomas, is typical of mycobacterial infections when a cellular-mediated immunity (CMI) is mounted (36, 44). As shown, in progressive murine infection by virulent M. ulcerans, such a switch is not fully completed. This observation correlates with the finding that progressive infection of the skin, disseminated disease, and osteomyelitis in BU patients were associated with an absence of granulomas in the infected areas (33, 49, 66). Conversely, with the noncytotoxic strain 5114, which is not virulent for mice and does not cause leukocyte death and necrosis of the subcutaneous tissue at any stage of infection, the acute neutrophilic profile of the cellular infiltrate gradually switches to a predominance of mononuclear cells and formation of granuloma-like structures. Not surprisingly, this type of chronic response was observed in BU patients during the healing phase of disease (30, 31, 40, 49, 66, 74).

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type of response (24, 25, 26, 55, 76), while a shift to a Th2 phenotype is related to susceptibility (26), and point to a protective effect of BCG vaccination (46, 53, 54, 63, 66, 73). Additionally, as BU disease progresses to healing, granuloma formation occurs (30, 31, 35, 40, 49, 66, 74), and the burulian skin test (65) tends to change from negative to positive (13, 42). In contrast, disseminated disease and osteomyelitis were reported to be associated with defects in granuloma formation (33, 39).

Taking into account that (i) CMI and delayed-type hypersensitivity occur not only in murine M. ulcerans infections but also in human BU; (ii) in the murine model, as shown in this study, extensive necrotic areas with extracellular bacilli that resemble the classical BU histopathology coexist, in advanced phases, with inflammatory infiltrates and intracellular bacilli; (iii) there are reports describing inflammatory infiltrates with intracellular bacilli in other animal models of infection; and (iv) a recent publication (29) reports the occurrence of infiltrates with neutrophils and mononuclear cells in 92% of 78 biopsies of confirmed BU, studies directed to the evaluation of the interactions between phagocytes and M. ulcerans in human infections are justified.

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FIG. 7. Schematic representation of the progression of BU. Panel A shows M. ulcerans (red bacilli) being phagocytosed in the initial stage of infection by neutrophils and macrophages that are present in the early acute inflammatory infiltrate. Panel B represents a more advanced infection stage characterized by the onset of cell destruction, showing predominant neutrophilic cell infiltrate, apoptotic neutrophils and macrophages, and cell debris resulting from postapoptotic secondary necrosis. Panel C highlights an advanced stage of BU with extensive acellular areas with numerous extracellular bacilli, tissue damage represented by accumulation of cellular fragments, continued predominance of neutrophils, and low number of macrophages and lymphocytes. This stage is reminiscent of what has been observed in biopsies of advanced BU. The orange-yellow gradient represents the production and spreading of M. ulcerans exotoxin.

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