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RESEARCH ARTICLE

Clofazimine Modulates the Expression of Lipid Metabolism Proteins in *Mycobacterium leprae*-Infected Macrophages

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Abstract

Mycobacterium leprae (M. leprae) lives and replicates within macrophages in a foamy, lipid-laden phagosome. The lipids provide essential nutrition for the mycobacteria, and *M. leprae* infection modulates expression of important host proteins related to lipid metabolism. Thus, *M. leprae* infection increases the expression of adipophilin/adipose differentiation-related protein (ADRP) and decreases hormone-sensitive lipase (HSL), facilitating the accumulation and maintenance of lipid-rich environments suitable for the intracellular survival of *M. leprae*. HSL levels are not detectable in skin smear specimens taken from leprosy patients, but re-appear shortly after multidrug therapy (MDT). This study examined the effect of MDT components on host lipid metabolism *in vitro*, and the outcome of rifampicin, dapsone and clofazimine treatment on ADRP and HSL expression in THP-1 cells. Clofazimine attenuated the mRNA and protein levels of ADRP in *M. leprae*-infected cells, while those of HSL were increased. Rifampicin and dapsone did not show any significant effects on ADRP and HSL expression levels. A transient increase of interferon (IFN)-β and IFN-γ mRNA was also observed in cells infected with *M. leprae* and treated with clofazimine. Lipid droplets accumulated by *M. leprae*-infection were significantly decreased 48 h after clofazimine treatment. Such effects were not evident in cells without *M. leprae* infection. In clinical samples, ADRP expression was decreased and HSL expression was increased after treatment. These results suggest that clofazimine modulates lipid metabolism in *M. leprae*-infected macrophages by modulating the expression of ADRP and HSL. It also induces IFN production in *M. leprae*-infected cells. The resultant decrease in lipid accumulation, increase in lipolysis, and activation of innate immunity may be some of the key actions of clofazimine.

Author Summary

Leprosy, caused by *Mycobacterium leprae (M. leprae)*, is an ancient infectious disease that remains the leading infectious cause of disability. After infection, *M. leprae* lives inside host macrophages that contain a large amount of lipids, which is thought to be an essential microenvironment for *M. leprae* to survive in host cells. *M. leprae* infection increases lipid accumulation in macrophages and decreases the metabolic breakdown of lipids (catabolism). In addition, the treatment of leprosy with multidrug therapy (MDT) reverses the effect of infection

on the modulation of lipid metabolism. We therefore aimed to use cultured human macrophage cells to determine which of the three MDT drugs (clofazimine, dapsone, or rifampicin) is responsible for this effect. We found that only clofazimine affects lipid accumulation and catabolism in *M. leprae*-infected cells *in vitro*. The amounts of lipids accumulated in the cells decreased when clofazimine was added to the cell culture medium. Clofazimine also activated immune responses in *M. leprae*-infected cells. These results suggest that the effectiveness of clofazimine against leprosy is due to the modulation of lipid metabolism and activation of immune reactions in *M. leprae*-infected host cells.

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INTRODUCTION

Leprosy is a chronic infectious disease caused by *Mycobacterium leprae* (*M. leprae*), which is a typical intracellular pathogen that parasitizes tissue macrophages (histiocytes) and Schwann cells of the peripheral nerves of the dermis. Although its prevalence has declined over the last several decades due to the introduction of multi-drug therapy (MDT) by the World Health Organization (WHO), leprosy remains a major public health problem in many developing countries: In 2010, 228,474 new cases were registered worldwide [1]. Based on their clinical, histological and immunological manifestations, leprosy patients are classified into five groups that comprise one continuous spectrum: Tuberculoid (TT), Borderline Tuberculoid (BT), Borderline (BB), Borderline Lepromatous (BL) and Lepromatous (LL) [2]. LL is characterized by widespread skin lesions containing numerous bacilli that live in the foamy or enlarged lipid-filled phagosome within macrophages. Schwann cells in LL nerves also have the foamy, lipid-laden appearance that favors mycobacterial survival and persistence. In Schwann cells, *M. leprae* infection-induced biogenesis of lipid droplets correlates with increased prostaglandin E2 (PGE2) and interleukin-10 (IL-10) secretion, which is essential for leprosy pathogenesis [3], [4]. Although lipid-laden macrophages

are also observed in other mycobacterial infections, including tuberculosis [5], [6], the amount of lipid and the number of infected macrophages are most prominent in cases of LL [7], [8].

The PAT protein family is named after three of its members: perilipin, adipophilin/adipose differentiation-related protein (ADRP), and tail-interacting protein of 47 kDa (TIP47). PAT family members are responsible for the transportation of lipids and the formation of lipid droplets in a variety of tissues and cultured cell lines, including adipocytes [9]–[12]. ADRP selectively increases the uptake of long chain fatty acids and has an essential role in fatty acid transport [13], [14]. Hormone-sensitive lipase (HSL), as the first enzyme identified in the induction of lipo-catabolic action initiated by hormones, is the predominant lipase effector of catecholamine-stimulated lipolysis in adipocytes [15]. Therefore, ADRP and HSL have opposing functions, i.e., lipid accumulation vs. its degradation. ADRP and HSL also play important roles in lipid accumulation in *M. leprae*-infected macrophages [8], [16]. *M. leprae* infection increased the expression of ADRP mRNA and protein, facilitating the accumulation and maintenance of a lipid-rich environment suitable for intracellular survival [8]. Conversely, HSL expression was suppressed in macrophages infected with *M. leprae* [16]. These results suggest that both ADRP and HSL influence the lipid-rich environment that favors *M. leprae* parasitization and survival in infected host cells. In our previous study, HSL expression was not detectable in slit-skin smear specimens from non-treated LL and BL patients, but it re-appeared shortly after MDT treatment [16]. However, how treatment modulates HSL expression is not clear. In the present study, we determine the effect of MDT components on host lipid metabolism by investigating the influence of rifampicin, dapsone and clofazimine on the expression of ADRP and HSL in THP-1 cells.

MATERIALS AND METHODS

Ethic statement

Human specimens were used according to the guidelines approved by the Ethical Committee of the National Institute of Infectious Diseases (Tokyo, Japan). All samples were anonymized before use.

Drugs

Clofazimine (Sigma-Aldrich Co., St. Louis, MO), rifampicin (Wako Pure Chemical Industries Ltd., Osaka, Japan) and dapsone (Wako Pure Chemical Industries Ltd.) were dissolved in dimethyl sulfoxide (DMSO) and stored at 4°C. The final concentration used in the culture medium was 8.0 μg/ml rifampicin, 5.0 μg/ml dapsone or 2.0 μg/ml clofazimine.

M. leprae isolation and cell culture

Hypertensive nude rats (SHR/NCrj-rnu), infected with the Thai53 strain of *M. leprae* [17], [18] were kindly provided by Dr. Y. Yogi of the Leprosy Research Center, National Institute of Infectious Diseases. Japan. The protocol was approved by the Experimental Animal Committee, of the National Institute of Infectious Diseases, Tokyo, Japan (Permit Number: 206055). Animal studies were carried out in strict accordance with the recommendations from Japan's Animal Protection Law. *M. leprae* was isolated as previously described [19], [20]. The human premonocytic cell line THP-1 was obtained from the American Type Culture Collection (ATCC; Manassas, VA). The cells were cultured in six-well plates in RPMI medium supplemented with 10% charcoal-treated fetal bovine serum (FBS), 2% non-essential amino acids, 100 IU/ml penicillin and 100 μg/ml streptomycin at 37°C in

5% CO_2 [7], [8]. Typically, 3×10^7 bacilli were added to 3×10^6 THP-1 cells (multiplicity of infection: MOI = 10).

RNA preparation and reverse transcription polymerase chain reaction (RT-PCR)

Total RNA from cultured cells was prepared using RNeasy Mini Kits (Qiagen Inc., Valencia, CA) as described previously [7], [8]. Total RNA preparation from slit-skin smear samples was performed as described [8], [16]. Briefly, stainless steel blades (Feather Safety Razor Co., Osaka, Japan) used to obtain slit-skin smear specimens were rinsed in 1 ml of sterile 70% ethanol. The tube was then centrifuged at 20,000×g for 1 min at 4°C. After removing the supernatant, RNA was purified with the same protocol that was used for cultured cells. The RNA was eluted in 20 µl of elution buffer and treated with 0.1 U/µl DNase I (TaKaRa Bio, Kyoto, Japan) at 37°C for 60 min to degrade any contaminating genomic DNA. All RNA samples had an OD260/280 of 1.8–2.0 and an OD260/230 >1.8. RNA sample quality was also confirmed using denaturing agarose gel electrophoresis and the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) (**Fig. S1**). Total RNA from each sample was reverse-transcribed to cDNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) with random primers [8], [16]. The following primers were used to amplify specific cDNAs: ADRP: 5′-TGTGGAGAAGACCAAGTCTGTG-3′ (forward) and 5′-GCTTCTGAACCAGATCAAATCC-3′ (reverse); HSL: 5′-CTCCTCATGGCTCAACTCCTTCC-3′ (forward) and 5′-AGGGGTTCTTGACTATGGGTG-3′ (reverse); interferon (IFN)-β: 5′-TGCTCTCCTGTTGTGCTTCTCCAC-3′ (forward) and 5′-CAATAGTCTCATTCCAGCCAGTGC-3′ (reverse); IFN-γ: 5′-GCAGAGCCAAATTGTCTCCTTTTAC-3′ (forward) and 5′-ATGCTCTTCGACCTCGAAACAGC-3′ (reverse) and actin: 5′-AGCCATGTACGTAGCCATCC-3′ (forward) and 5′-TGTGGTGGTGAAGCTGTAGC-3′ (reverse). Touchdown PCR was performed using a PCR Thermal Cycler DICE (TaKaRa Bio, Tokyo, Japan) [7], [8]. Briefly, the PCR mixture was first denatured for 5 min at 94°C, followed by 20 cycles of three-temperature PCR consisting of a 30-sec denaturation at 94°C, a 30-sec annealing that started at 65°C and decreased 0.5°C every cycle to 55°C, and a 45-sec extension at 72°C. An additional 10 cycles were performed for ADRP and β-actin, and 14 cycles for HSL with a fixed annealing temperature of 55°C. The products were analyzed by 2% agarose gel electrophoresis.

Protein preparation and Western blot analysis

Cellular protein was extracted and analyzed as previously described [16], [21]. Briefly, cells were lysed in a lysis buffer containing 50 mM HEPES, 150 mM NaCl, 5 mM EDTA, 0.1% NP40, 20% glycerol, and protease inhibitor cocktail (Complete Mini, Roche, Indianapolis, IN) for 1 h. After centrifugation, the supernatant was transferred and 10 µg of protein was used for analysis. Cellular proteins were mixed with 4× LDS sample buffer and 10× reducing agent (Invitrogen, Life Technologies, Carlsbad, CA) and incubated for 10 min at 70°C prior to electrophoresis. Proteins were separated on NuPage 4–12% Bis Tris Gels and transferred using an iBlot Gel Transfer Device (Invitrogen). The membrane was washed with PBST (phosphate buffered saline (PBS) with 0.1% Tween 20), blocked in blocking buffer (PBST containing 5% skim milk) overnight, and then incubated with either rabbit anti-ADRP antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA; 1:2,000 dilution), rabbit anti-HSL antibody (Cell Signaling Technology, Danvers, MA; 1:1,000 dilution) or goat anti-β-actin antibody (Santa Cruz; dilution 1:2,000). After washing with PBST, the membrane was incubated for 1 h with biotinylated donkey anti-rabbit antibody for ADRP and HSL (GE Healthcare, Fairfield, CT; 1:2,000 dilution) or biotinylated donkey anti-goat antibody for β-actin

(Millipore, Billerica, MA; dilution 1:10,000) followed by streptavidin-HRP (GE Healthcare; 1:10,000 dilution) for 1 h. The signal was developed using ECL Plus Reagent (GE Healthcare).

Lipid staining

THP-1 cells were grown on glass coverslips in 24-well plates for 24 h, before the culture medium was exchanged with RPMI 1640 containing *M. leprae* and clofazimine. Control and drug-treated THP -1 cells were fixed in 10% formalin for 10 min. They were then washed with Dulbecco's PBS (DPBS) and balanced with 60% isopropanol for 1 min before staining with oil-red-O (Muto Pure Chemicals, Tokyo, Japan) for 10 min. The cells were counterstained with hematoxylin for 5 min followed by ethanol dehydration and coverslip sealing.

Immunohistochemistry

Archived formalin-fixed, paraffin-embedded tissue sections were subjected to immunohistochemical staining as described [7]. Briefly, deparaffinized sections were heated in 1 mM NaOH at 120°C for 5 min for antigen retrieval. They were then washed with PBST and blocked in blocking buffer (DAKO, Carpinteria, CA) for 10 min, and then incubated with either anti-ADRP antibody (Santa Cruz Biotechnology Inc.; 1:200 dilution) or anti-HSL antibody (Cell Signaling Technology; 1:100 dilution), for 1 h at room temperature. After washing the slides with PBST, peroxidase-labeled streptavidin-biotin method was employed using the LSAB2 kit (DAKO) and 3,3-diaminobenzidine tetrahydrochloride (DAB) for the staining of ADRP. Tyramide signal amplification (TSA)-HRP method was utilized to amplify HSL staining signals using the TSA Biotin System (PerkinElmer, Inc., Waltham, MA) according to the manufacturer's protocol. Sections were then stained using carbol fuchsin to visualize acid-fast mycobacteria and counterstained with hematoxylin.

Others

All experiments were repeated at least three times. Since the replicates produced essentially the same outcomes, representative results from these independent experiments are shown in the figures.

RESULTS

Clofazimine decreases ADRP and increases HSL mRNA levels in macrophages infected with *M. leprae*

The effect of MDT drugs on lipid metabolism in *M. leprae*-infected macrophages was examined by infecting human premonocytic THP-1 cells with *M. leprae* (MOI = 10) in the presence of 8.0 μg/ml rifampicin, 5.0 μg/ml dapsone or 2.0 μg/ml clofazimine for 24 h. Total RNA was isolated and RT-PCR analysis was performed to evaluate possible changes in ADRP and HSL mRNA levels. In our previous studies, *M. leprae* infection has been shown to increase ADRP and decrease HSL expression, which will in turn increase the lipid accumulation that is thought to contribute to maintaining a phagosome environment which permits *M. leprae* to parasitize tissue macrophages [8], [16]. However, when *M. leprae*-infected THP-1 cells were treated with clofazimine, ADRP expression levels decreased and HSL expression increased (**Fig. 1**). Rifampicin and dapsone did not show significant effects on the mRNA expression of ADRP, while they decreased HSL expression by augmenting the effect of *M. leprae* infection.

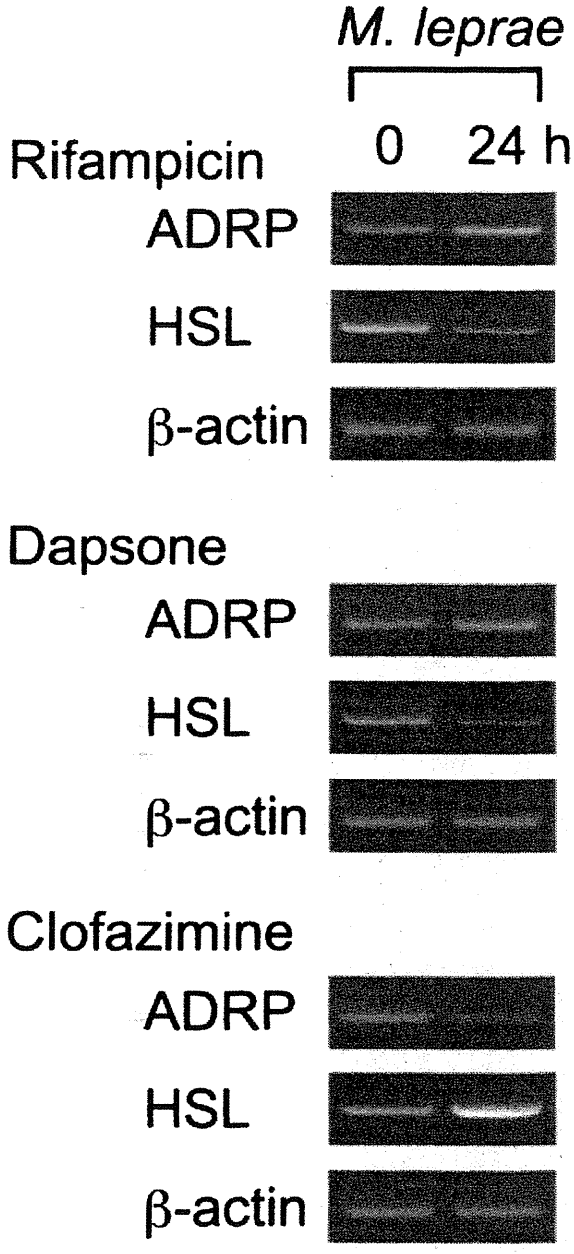

Figure 1. Expression of ADRP and HSL is modulated by clofazimine in THP-1 cells infected with *M. leprae*.

THP-1 cells were cultured in six-well plates with culture medium containing either 8.0 μg/ml rifampicin, 5.0 μg/ml dapsone or 2.0 μg/ml clofazimine with *M. leprae* infection (MOI = 10). After incubating for 24 h, total RNA was purified and RT-PCR analysis of ADRP, HSL and β-actin was performed. Representative results from three independent experiments are shown.

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The effect of clofazimine is specific only for *M. leprae*-infected cells

To further evaluate the effect of clofazimine on ADRP and HSL expression, THP-1 cells were treated with clofazimine in the presence or absence of *M. leprae* infection for 6, 12, 24 and 48 h. Total RNA and cellular protein were extracted and used for RT-PCR analysis and Western blot analysis, respectively. Linearity of the RT-PCR amplifications of ADRP, HSL and β-actin was confirmed by serial dilution of RNA samples and densitometric analysis of the bands (Fig. S2). RT-PCR showed that clofazimine alone had no effect on ADRP and HSL mRNA levels in control THP-1 cells (Fig. 2, left panel). Consistent with previous reports, ADRP mRNA expression was increased and HSL mRNA expression was decreased when THP-1 cells were infected with *M. leprae* (Fig. 2, middle panel) [8],

[16]. However, simultaneous clofazimine treatment and *M. leprae* infection of THP-1 cells led to decreased ADRP and increased HSL mRNA levels (Fig. 2, right panel). The decrease of ADRP and increase of HSL mRNA expression were further confirmed by quantitative real-time PCR (Fig. S3), which also supports the linearity of our RT-PCR data. Thus, it was shown that clofazimine modulated expression of ADRP and HSL only in *M. leprae*-infected cells. Similar results were observed for ADRP and HSL protein expression levels in each experiment.

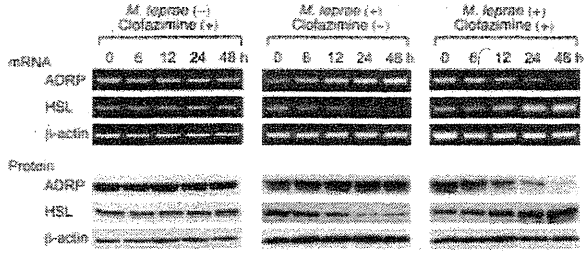

Figure 2. Only *M. leprae*-infected THP-1 cells are susceptible to clofazimine.

THP-1 cells were cultured in six-well plates with or without 2.0 μg/ml clofazimine in the presence or absence of *M. leprae* infection (MOI = 10). After incubating for the indicated period of time, total RNA and total cellular protein were purified and RT-PCR and Western blot analyses of ADRP, HSL and β-actin were performed. Representative results from three independent experiments are shown.

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Clofazimine antagonizes the effects of *M. leprae* to modulate ADRP and HSL expression

In the above studies, THP-1 cells were simultaneously treated with clofazimine and infected with *M. leprae*. Therefore, there was a possibility that clofazimine might have modulated the cellular environments of THP-1 cells before engulfing *M. leprae*. To eliminate this possibility and to imitate clinical situations, THP-1 cells were first infected with *M. leprae* for 24 h, to allow cells to engulf enough bacilli, before they were treated with clofazimine. *M. leprae* infection enhanced ADRP expression and suppressed HSL expression for up to 72 h (Fig. 3, left panel), which is consistent with the results shown in Fig. 2, middle panel. However, adding clofazimine 24 h after *M. leprae* infection produced lower levels of ADRP expression, but increased HSL expression (Fig. 3, right panel). Interestingly, ADRP expression fell even lower than the original level, and HSL rose higher than original levels, following clofazimine treatment. These results suggest that the lipid catabolic activity once suppressed by *M. leprae* infection was reactivated by clofazimine treatment, which in turn would promote lipolysis in infected macrophages and decrease cellular lipids. Also, these results are consistent with clinical situations in which HSL mRNA levels were recovered following successful treatment with MDT in LL and BL patients [16].

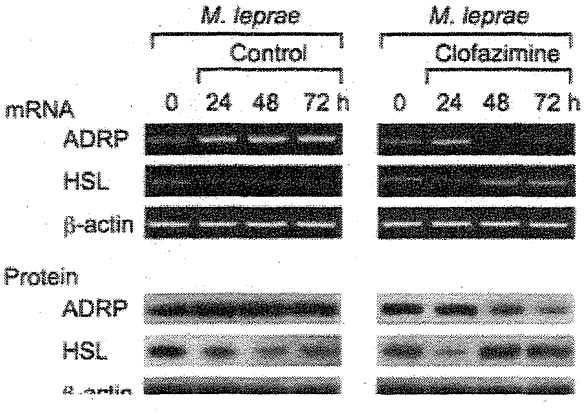

Figure 3. Clofazimine counteracts *M. leprae* to modulate ADRP and HSL expression levels.

THP-1 cells were cultured in six-well plates and infected with *M. leprae* (MOI = 10) for 24 h. Clofazimine (2.0 μg/ml) was added and incubation continued another 24 and 48 h (48 and 72 h from *M. leprae* infection). Total RNA and total cellular protein were purified and RT-

PCR and Western blot analyses of ADRP, HSL and β-actin were performed. Representative results from three independent experiments are shown.

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Clofazimine increases expression of IFN-β and IFN-γ mRNA in *M. leprae*-infected THP-1 cells

The decrease in ADRP expression and increase in HSL expression produced by clofazimine treatment were also observed when *M. leprae*-infected cells were further treated with peptidoglycan (PGN), a ligand for Toll-like receptor (TLR)-2, to activate innate immunity [8], [16]. We therefore hypothesized that clofazimine treatment might activate the innate immune response of THP-1 cells, which also confers bactericidal activities. To assess activation of innate immunity, production of interferon IFN-β and IFN-γ mRNA was evaluated in control and *M. leprae*-infected THP-1 cells treated with clofazimine. A transient increase of IFN-β and induction of IFN-γ were observed only in THP-1 cells infected with *M. leprae* and treated with clofazimine (Figs. 4A and 4B). Transient induction of IFNs as a result of macrophage activation is consistent with previous reports [22]–[24]. Innate immune activation of infected cells will further contribute to the elimination of intracellular bacilli, which is also consistent with the observation that the active form of vitamin D suppresses CORO1A expression in THP-1 cells [21].

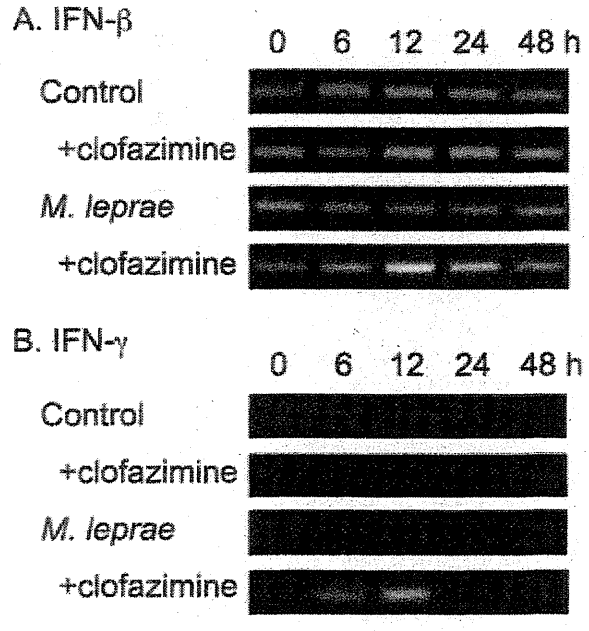

Figure 4. Clofazimine increases mRNA expression of IFN-β and IFN-γ in *M. leprae* -infected THP-1 cells.

THP-1 cells were cultured in six-well plates with or without 2.0 μg/ml clofazimine in the presence or absence of *M. leprae* infection (MOI = 10). After incubating for the indicated period of time, total RNA was purified and RT-PCR analysis of IFN-β (A) and IFN-γ (B) was performed. Representative results from three independent experiments are shown.

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Clofazimine treatment decreases the cellular lipid droplets in *M. leprae*-infected THP-1 cells

To test whether the decrease in ADRP expression and increase in HSL expression after clofazimine treatment would result in less accumulation of cellular lipids after *M. leprae* infection, THP-1 cells were infected with *M. leprae* (MOI = 10) in the presence or absence of 2.0 μg/ml clofazimine for 48

h. Oil-red-O staining clearly demonstrated the accumulation of cellular lipid droplets following *M. leprae* infection (**Fig. 5B** vs. **Fig. 5A**). In *M. leprae*-infected cells treated with clofazimine, the amount of lipid droplets in the cell had significantly decreased by 48 h (**Fig. 5C** vs. 5B). The decrease in cellular lipid droplets is in agreement with the results shown in this study in which clofazimine decreased ADRP and increased HSL expression in *M. leprae*-infected cells.

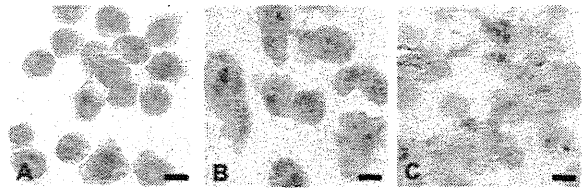

Figure 5. Clofazimine decreases cellular lipid accumulation in *M. leprae*-infected THP-1 cells.

THP-1 cells were grown on glass coverslips in 24-well plates. Cells with no treatment (A), infected with *M. leprae* (MOI = 10) (B), and infected with *M. leprae* (MOI = 10) and treated with clofazimine (2.0 μg/ml) (C) were cultured for 48 h. Oil-red-O staining followed by brief hematoxylin counter staining was performed and observed under a microscope. Representative results from three independent experiments are shown. Bars = 10 μm.

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ADRP and HSL expression levels in skin lesions correlate with the clinical course of leprosy before and after treatment of leprosy patients

To confirm the expression pattern of ADRP and HSL in clinical courses of leprosy, ADRP and HSL mRNA levels were evaluated in slit-skin smear specimens by RT-PCR analysis. ADRP mRNA was detected in all LL and most BL cases tested (**Fig. 6A**, right panel). HSL mRNA was detected in four BL cases; however, ADRP mRNA expression in these cases was absent or weaker than in other BL samples (**Fig. 6A**, cases 2, 4, 6 and 8). In one case, from which serial samples were obtained, the expression of ADRP mRNA decreased and HSL mRNA levels increased after treatment (**Fig. 6B**).

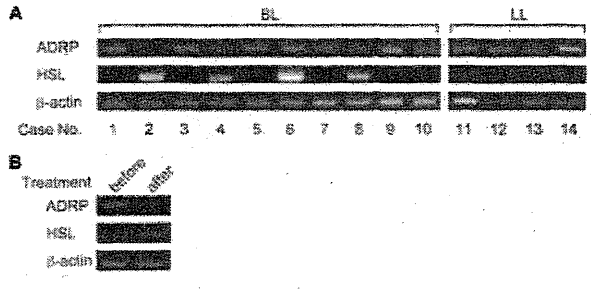

Figure 6. Detection of ADRP and HSL mRNA in slit-skin smear samples from leprosy patients.

Total RNA was isolated from slit-skin smear specimens taken from ten BL and four LL patients (A) or from one patient before and after treatment (B). Total RNA was purified and RT-PCR analysis of ADRP, HSL and β-actin was performed. Representative results from three independent experiments are shown.

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To further confirm changes in ADRP and HSL expression following treatment, immunohistochemical and acid-fast staining were performed using formalin-fixed paraffin-embedded skin tissue sections. Consistent with a previous report, ADRP localized to phagosome membranes that contains solid-shaped *M. leprae* (**Fig. 7A**) [8]. HSL staining was not evident before treatment (**Fig. 7C**). Three

months after treatment, staining of the bacilli showed a dotted pattern with no solid-staining, indicating degeneration of *M. leprae* (Fig. 7 B and 7D). At this point, ADRP staining was faint (Fig. 7B), but strong HSL staining was observed along the phagosomal membrane (Fig. 7D). These staining patterns correlate with changes in mRNA levels of ADRP and HSL in the skin smears (Fig. 6B).

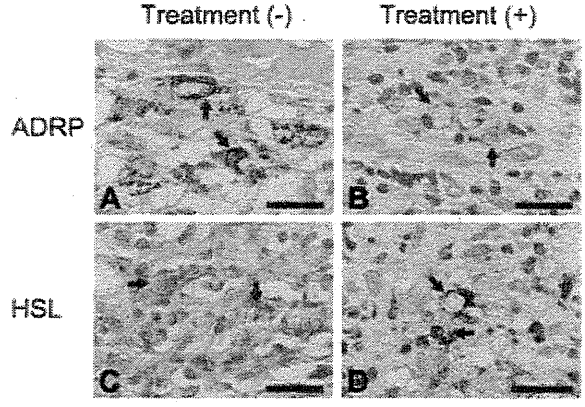

Figure 7. Immunostaining of ADRP and HSL proteins in skin biopsy specimens before and after treatment.

Sections of skin biopsy specimens taken from one patient before (A and C) and after (B and D) treatment were subjected to immunostaining of ADRP (A and B) and HSL (C and D), followed by acid-fast staining for *M. leprae* and hematoxylin counterstaining. Arrows indicate phagosome membrane that contains *M. leprae*. Representative results from three independent experiments are shown. Bars = 20 μm.

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DISCUSSION

In previous studies, we showed that *M. leprae* infection increases ADRP expression and decreases HSL expression in host macrophages [8], [16]. The results of the present study demonstrate that clofazimine, one of the three major drugs used to treat leprosy, counteracts the effect of *M. leprae* to reduce ADRP and increase HSL expression of both mRNA and protein levels. These results are consistent with our observations in clinical samples obtained from leprosy patients, in which HSL levels were not detectable in skin smear specimens before treatment, but re-appeared shortly after MDT [8], [16]. The other two MDT drugs, dapsone and rifampicin, revealed no effects on the expression of either ADRP or HSL.

Mycobacteria survive by evading the host immune system and accessing host metabolic pathways to obtain nutrients for growth. *M. leprae* has undergone reductive evolution and pseudogenes now occupy half of its genome [25]–[27], thus *M. leprae* is thought to be the mycobacterium most dependent on host metabolic pathways, including host-derived lipids. As we previously reported, PGN can activate TLR2 to increase the expression of HSL [16] and suppress ADRP and perilipin expression [7], [8], [21]. These effects mediated by the TLR-initiated signaling pathway will induce lipid degradation, which makes it difficult for *M. leprae* to survive within host cells. *M. leprae* infection not only suppresses HSL expression, but also invalidates all effects of PGN on ADRP and perilipin, thus ensuring a phagosome environment that is favorable for mycobacterial survival [16]. In the present study clofazimine increased HSL expression and decreased ADRP expression only in *M. leprae*-infected cells. The amounts of lipids accumulated in the cells decreased when clofazimine was added to the cell culture medium. The decrease of the lipid-rich environment against the survival of *M. leprae* may be one of the key actions of clofazimine.

Clofazimine was the first clinically developed riminophenazine for the treatment of tuberculosis [28]. Its use has been extended to many Gram-positive bacterial infections as well as mycobacterial diseases [28]–[30]. The drug is now widely used for the treatment of leprosy, but its mechanism remains unclear [31]–[33]. The drug is extremely lipophilic and is also active in membrane destabilization and possible promotion of antigen processing. Stimulated phospholipase A2 activity and subsequent accumulation of arachidonic acid and lysophospholipids were confirmed in clofazimine-induced membrane destabilization [29], [34]. Increased major histocompatibility complex (MHC) class II expression in peripheral blood monocytes [35], up-regulated lysosomal enzyme activity of cultured macrophages [36] and decreased suppressor T-cell activity in mycobacteria-infected mice [37] reveal the potential role of clofazimine in facilitating immune recognition.

Although the underlying molecular mechanisms are not clear, clofazimine suppressed ADRP and induced HSL, IFN-β and IFN-γ expression only in cells infected with *M. leprae*, the same effects products by PGN [8], [16], [21]. Therefore, it is possible that clofazimine revives at least some of the activities of PGN, which is normally shielded by redundant mycolic acid at the *M. leprae* cell wall. Given the extreme lipophilicity of clofazimine and its activity against many Gram-positive bacteria, clofazimine may interact with the mycolic acid in the *M. leprae* cell wall that facilitates the exposure of PGN, which in turn activates TLR2-mediated signaling cascades, subsequently decreasing ADRP and increasing HSL [8], [16], [21]. Furthermore, since most lepra reactions, a cell -mediated, delayed-type hypersensitivity immune response, occur during or after MDT [38], [39], the prospect that clofazimine rescues shielded PGN activities, promoting lysosomal fusion and antigen processing, would be a plausible explanation for the trigger of lepra reactions.

The results from present and previous studies may explain the underlying mechanisms, at least in part, of successful parasitization of *M. leprae* and the effects of MDT treatment observed in patients. In conclusion, we have shown that clofazimine devastates the lipid-rich environment in *M. leprae*-infected host macrophages by modulating the expression of ADRP and HSL and activates the innate immune response of infected cells, both of which would be important in fighting mycobacterial infection.

SUPPORTING INFORMATION

Figure S1.

Quality of RNA samples purified from THP-1 cells infected with *M. leprae*. RNA samples were purified from THP-1 cells infected with *M. leprae* as described in the Materials and Methods. Ten samples were analyzed using a 1% denatured agarose gel (A) and four were analyzed with theAgilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) (B).

(EPS)

Figure S2.

Linearity of RT-PCR analysis. RNA samples were serially diluted and RT-PCR analysis of ADRP, HSL and β-actin was performed. Specific bands on the agarose gel were quantified using ImageJ64 software.

(EPS)

Figure S3.

The effect of simultaneous clofazimine treatment and *M. leprae* infection on mRNA levels in THP-1 cells. THP-1 cells were cultured in six-well plates with or without 2.0 μg/ml clofazimine in the presence of *M. leprae* infection (MOI = 10). After incubating for the indicated period of time, total RNA was purified and real-time PCR analyses of ADRP (A), HSL (B) and β-actin were performed as previously described (reference 8). The same primers that were used for RT-PCR analysis were utilized with SYBER Green PCR Master Mix (Applied Biosytems). All samples were amplified in triplicate from the same RNA preparation. Each result is expressed as the mean ± SE. The Student's t-test was used for statistical analysis. One asterisk indicates a value of $P<0.05$; two asterisks indicate a value of $P<0.01$; and three asterisks indicate a value of $P<0.001$.

(EPS)

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: YD KS. Performed the experiments: YD TA TH KT YI. Analyzed the data: YD KS. Contributed reagents/materials/analysis tools: MG MM NI. Wrote the paper: YD KS.

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REVIEW ARTICLE

Current status of leprosy: Epidemiology, basic science and clinical perspectives

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ABSTRACT

Leprosy has affected humans for millennia and remains an important health problem worldwide, as evidenced by nearly 250 000 new cases detected every year. It is a chronic infectious disorder, caused by *Mycobacterium leprae*, that primarily affects the skin and peripheral nerves. Recent advances in basic science have improved our knowledge of the disease. Variation in the cellular immune response is the basis of a range of clinical manifestations. The introduction of multidrug therapy has significantly contributed to a decrease in the prevalence of the disease. However, leprosy control activities, including monitoring and prevention programs, must be maintained.

Key words: diagnosis, disability, leprosy, *Mycobacterium leprae*, social stigma.

INTRODUCTION

Leprosy, or Hansen's disease, is a chronic infectious disease caused by the acid-fast bacterium *Mycobacterium leprae*. Norwegian physician Gerhard Armauer Hansen identified the bacillus in the patients in 1873, making leprosy the first disease ascribed to a bacterial origin. Leprosy usually affects the dermis of the skin and peripheral nerves, but has a wide range of clinical manifestations. It can be progressive and cause permanent damage if left without treatment. Divided into paucibacillary (TB; tuberculoid pole) or multibacillary (MB; lepromatous pole), depending on the bacillary load, the disease manifests first in discoloration of the skin and then in rashes and nodules. The introduction of dapsone (diphenyl sulfone, DDS) in 1941 brought the first effective therapy, and multidrug therapy (MDT) was introduced by the World Health Organization (WHO) in 1981 to limit the development of drug resistance. Endemic leprosy has declined markedly and the disease is now rare in most industrialized countries. It is still a major public health problem in developing countries, where hundreds of thousands of new cases are diagnosed each year. In many of these countries, social stigmatization is an additional burden. Therefore, it is important that control activities continue if the disease burden and damaging impacts of leprosy are to be reduced. Dermatologists should be familiar with leprosy and other diseases needed for differential diagnosis.

EPIDEMIOLOGY

The WHO publishes an annual report on the worldwide incidence of leprosy, including the number of new cases, prevalence and disabilities.[1] The detection of new cases by the WHO has declined from 514 718 in 2003 to 244 796 in 2009, but the rate of decrease is getting smaller each year. Among 244 796 new cases in 2009, 16 countries that reported 1000 or more new cases accounted for 93% of the total. These countries and the number of cases detected in 2009 are: India (133 717 cases), Brazil (37 610 cases), Indonesia (17 260 cases), Bangladesh (5239 cases), the Democratic Republic of the Congo (5062 cases), Ethiopia (4417 cases), Nepal (4394 cases), Nigeria (4219 cases), Myanmar (3147 cases), the United Republic of Tanzania (2654 cases), Sudan (2100 cases), Sri Lanka (1875 cases), the Philippines (1795 cases), China (1597 cases), Madagascar (1572 cases) and Mozambique (1191 cases).

The proportion of new cases with multibacillary leprosy ranged from 32.70% in the Comoros in Africa to 95.04% in the Philippines. The proportion of females among newly detected cases ranged from 6.50% in Ethiopia to 59.11% in the Central African Republic. The proportion of children among new cases ranged from 0.60% in Argentina to 30.30% in Papua New Guinea. Grade 2 disabilities in new cases ranged from 1.45% in Liberia to 22.8% in China. As the number of new cases declines, the damaging

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impact of the disease on the physical, social and economic well-being of individuals and families affected by leprosy are also expected to decline.

Very few new leprosy patients are registered in developed countries. When leprosy is detected, it is primarily found among immigrants from countries where the disease is still endemic. There is an association between the incidence of leprosy and socioeconomic factors such as gross national product (GNP), personal housing expenditures and the number of persons per household, suggesting that improvements in socioeconomic conditions greatly contribute to the reduction of leprosy.[2] The proportion of children under the age of 15 years among newly detected cases would be a good indicator of the situation in a country/region. Similarly, the proportion of cases with grade 2 and visible disabilities among newly detected cases would be a reflection of early detection and treatment.

BACTERIOLOGY AND GENOMICS

Mycobacterium leprae is an obligate intracellular parasite that cannot be cultivated *in vitro*. It grows very slowly with an approximate generation time of 12–14 days. The inability to cultivate *in vitro* and the lack of animal models have been major disadvantages for leprosy research. However, the availability of the *M. leprae* genome sequence has contributed to knowledge of the disease. The first genome sequence of *M. leprae*, completed in 2001,[3] revealed that only half of the small genome contains protein-coding genes, while the remainder consists of pseudogenes and non-coding regions (Fig. 1). The number of pseudogenes is much larger in the *M. leprae* genome than in other mycobacteria,[4] and the number and proportion are exceptionally large in comparison with other pathogenic and non-pathogenic bacteria and archaea.[5,6] Many of the *M. leprae* pseudogenes are the result of stop codon insertions thought to be caused by the dysfunction of sigma factors or the insertion of repetitive sequences derived from transposons.[7–9] Despite this genetic damage, a specialized intracellular environment free from evolutionary competition has allowed the organism to survive.[3,10,11] It has been speculated that *M. leprae* has lost over 1500 genes from its genome and that non-coding regions are functionally silent and useless.[12] However, analyses have demonstrated that some of the pseudogenes and non-coding regions are highly expressed at the RNA level, and that expression of these RNA in clinical samples shows varying patterns among patients, suggesting as yet unknown functions.[13–16]

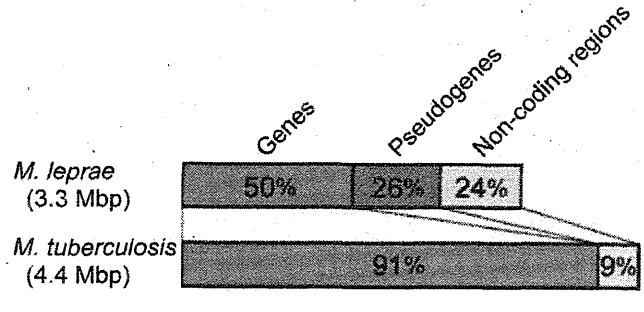

Figure 1. Only half of the *Mycobacterium leprae* genome contains functional genes. The percentage of functional genes, pseudogenes and non-coding regions are illustrated for *M. leprae* and *Mycobacterium tuberculosis* genomes.

Single nucleotide polymorphisms (SNP) and short or variable number tandem repeats have been used for *M. leprae* genotyping. SNP analysis revealed four primitive subtypes of *M. leprae* and the number is increasing as the analysis progresses.[17–19] Some reports have also presented the possibility of dual infections or phenotypically distinct strains of *M. leprae*; however, these situations are still somewhat obscure.[20,21]

TRANSMISSION AND PATHOLOGY

It is evident that humans are the major reservoir of *M. leprae* infection, while naturally occurring infection has been reported in wild animals, including the nine-banded armadillo and several species of primates.[22–32] A recent study found that the same genotypic strain of *M. leprae* was detected at high incidence in wild armadillos and leprosy patients in the southern USA, suggesting that leprosy may be a zoonosis in regions in which armadillos serve as a reservoir.[33]

Although transmission of *M. leprae* is not entirely understood, it is thought that long-term exposure of the respiratory system to airborne droplets is the main route of infection.[34,35] *M. leprae* is not very virulent, meaning that most people affected with leprosy are non-infectious, probably because the bacilli remain within the infected cells. Multibacillary patients, however, excrete *M. leprae* from their nasal mucosa and skin.[36] Close and repeated contact with these patients is also a source of transmission. Upon MDT treatment, however, the patients rapidly lose infectivity.

Even if infected, a long incubation period is required before clinical manifestation. The long incubation period of leprosy was demonstrated by an SNP analysis of an *M. leprae* genome derived from one of four spontaneous leprosy cases in chimpanzees. The chimpanzee was infected with *M. leprae* during infancy in West Africa, but the pathogenic signs of leprosy did not appear for at least 30 years.[30]

Mycobacterium leprae primarily infects histiocytes (or tissue macrophages) in the dermis and Schwann cells in the peripheral nerves. The unique tropism for peripheral nerves can lead to deformities even after the pathogen is successfully treated. The outcome of infection and clinical manifestation depend on the cellular immunity of the host, which is the first line of defense against *M. leprae* infection. There is a relationship between clinical manifestation and cytokine profiles within the skin lesions. T-helper cell (Th)1 cytokines, such as interleukin (IL)-2 and γ-interferon, play important roles in cellular immune responses in paucibacillary leprosy. Th2 cytokines, including IL-4, IL-5 and IL-10, augment humoral immune responses and predominate in multibacillary leprosy. Thus, there is an inverse correlation in the cytokine profiles that create the basis of paucibacillary and multibacillary leprosy.

Mycobacterium leprae should be recognized by the innate immune system and phagocytized by host macrophages. Toll-like receptor (TLR)2, in conjunction with TLR1, recognizes the cell wall lipids of *M. leprae* and subsequently activates innate immune responses.[37,38] However, some bacilli escape this initial attack of innate immunity and successfully parasitize the phagosome of macrophages. CORO1A, an actin-binding scaffold protein in the cell membrane of host cells, inhibits the phagosome/lysosome fusion, thereby helping the pathogen escape digestion.[38–40]

Mycobacterium leprae parasitization of macrophages occurs in a foamy or enlarged phagosome filled with lipids.[40,41] Because it is aerobic, it may survive in a granuloma environment with a relatively low oxygen tension gradient using lipids and fatty acids as carbon sources.[42] *M. leprae* creates a lipid-rich phagosome environment that is favorable for its survival.[43] Adipose differentiation-related protein (ADRP) and perilipin expression, which contribute to lipid intake, significantly increase following *M. leprae* infection. Infection also has a pronounced effect on Schwann cell lipid homeostasis via regulation of lipid droplet biogenesis and traffic, which favors *M. leprae* intracellular survival.[44]

It was long thought that leprosy might have a strong host genetic component. With the use of gene expression profiling, gene expression patterns associated with host immune response in lesions of human leprosy have been clarified.[45] Genes belonging to the leukocyte immunoglobulin-like receptor (LIR) family were significantly upregulated in lesions of lepromatous patients suffering from the disseminated form of the infection.[45] A genome-wide search for loci affecting the susceptibility to leprosy mapped a susceptibility locus to chromosome 6q25-q26.[46] There is a close relationship between leprosy susceptibility and SNP in the genes encoding tumor necrosis factor (TNF)-α and IL-10.[47]

CLINICAL FEATURES

Leprosy is a systemic disease that primarily affects the skin, nerves and eyes. *M. leprae* infection induces diverse clinical manifestations depending on the host immune responses. Paucibacillary leprosy is a milder disease characterized by few (≤5) hypopigmented, anesthetic skin lesions. The multibacillary form is associated with multiple (>5) skin lesions, nodules, plaques, thickened dermis or skin infiltration, and in some instances, involvement of the nasal mucosa, resulting in nasal congestion and epistaxis. The involvement of certain peripheral nerves may also be noted. In most cases of both paucibacillary and multibacillary disease, the diagnosis is straightforward. However, the small proportion of suspected cases that do not exhibit anesthetic patches require examination by a specialist to find other cardinal signs of the disease, including nerve involvement and a positive laboratory test for acid-fast bacilli.

Patients commonly present with weakness or numbness as the result of a peripheral-nerve lesion, or a burn or ulcer in an anesthetic hand or foot. In typical multibacillary leprosy, diffuse infiltration of the skin is evident. There may be many lesions that are not hypoaesthetic, while only a few hypopigmented lesions with reduced sensation are seen in paucibacillary patients. Careful inspection of the entire body is important. The great auricular nerve, ulnar nerve, median nerve, radial-cutaneous nerve, posterior tibial nerve and lateral popliteal nerve are frequently involved with enlargement, with or without tenderness, and standard regional patterns of sensory and motor loss.[48] Neuritic leprosy in India and Nepal is characterized by asymmetrical involvement of peripheral nerve trunks without visible skin lesions.[49–51]

The Ridley–Jopling classification system,[52] based on the *M. leprae*-specific immunological resistance status of the host, is clinically relevant and widely used, although the WHO only distinguishes between paucibacillary and multibacillary for simplicity of use in endemic countries. Ridley–Jopling divided the disease into six categories based on dermatological, neurological and histopathological findings: indeterminate (I), tuberculoid (TT), borderline tuberculoid (BT), mid-borderline (BB), borderline lepromatous (BL) and lepromatous (LL) (Fig. 2). TT leprosy can be associated with rapid and severe nerve damage, whereas LL is associated with chronicity and long-term complications. Borderline disease is unstable and can be complicated by lepra reactions as described in the "Lepra Reactions" section.

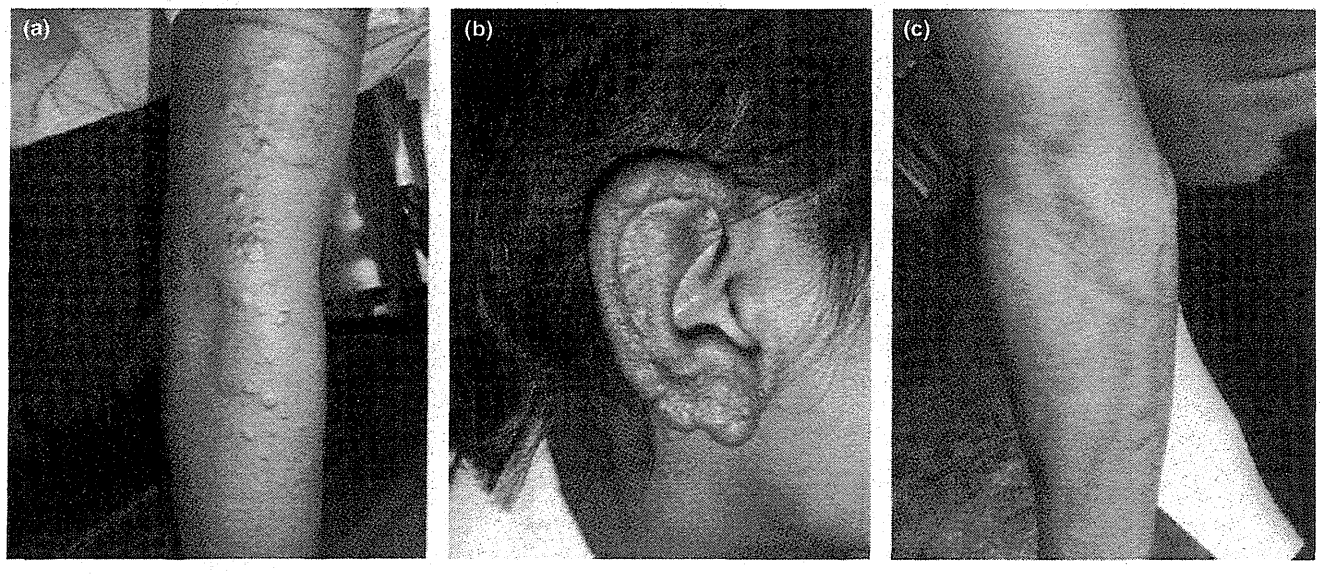

Figure 2. Typical dermatological views of leprosy patients. A multibacillary case (lepromatous) showing multiple nodules in the arms (a) and ears (b), and a paucibacillary case (borderline tuberculoid) with large erythema annulare, with discoloration in the middle of the lesion accompanied by loss of sensation (c).

DIAGNOSIS AND LABORATORY TESTS

Leprosy exerts systemic effects in addition to skin lesions, which is evident in the infiltration of bacilli into the nasal mucosa, bones and other organs of multibacillary patients.[53] Severe skeletal lesions, the hallmark of lepromatous leprosy, have been observed in excavated skeletal remains,[54–58] and *M. leprae* DNA has been isolated from such lesions (Fig. 3).[59] Eye damage is frequently seen in multibacillary patients resulting from both nerve damage and direct bacillary invasion.[60] Typically, lagophthalmos is caused by involvement of the zygomatic and temporal branches of the facial nerve. Other facial nerve damage, such as involvement of the ophthalmic branch of the trigeminal nerve, causes anesthesia of the cornea and conjunctiva, resulting in dryness and the risk of ulceration.

A diagnosis of leprosy is made based on cardinal signs such as hypopigmented or reddish patches with definite loss of sensation, thickened peripheral nerves and acid-fast bacilli in slit-skin smears or biopsy materials.[61,62] Smear and biopsy samples are subjected to acid-fast staining in addition to conventional histopathological diagnosis in order to demonstrate the presence of mycobacterium; however, bacilli are not usually detected in paucibacillary cases. The presence of neural inflammation is a histological characteristic of leprosy that can differentiate it from other granulomatous disorders. The polymerase chain reaction (PCR) is a sensitive method for the detection of *M. leprae* DNA that is widely used for differential diagnosis in advanced countries, although it cannot determine if viable organisms are present because DNA can persist long after microorganisms are dead.[15,30,59,63] Serum antibodies against *M. leprae* phenolic glycolipid-I (PGL-I) are found in multibacillary patients and some household contacts, although its specificity is relatively low.[30,64–66] Non-endemic countries do not usually consider leprosy during the differential diagnosis of skin lesions; however, it should be considered in a case of peripheral neuropathy or persistent skin lesions if patients are from endemic countries. Late diagnosis leads to continued transmission and increased risk of disability.[67,68]

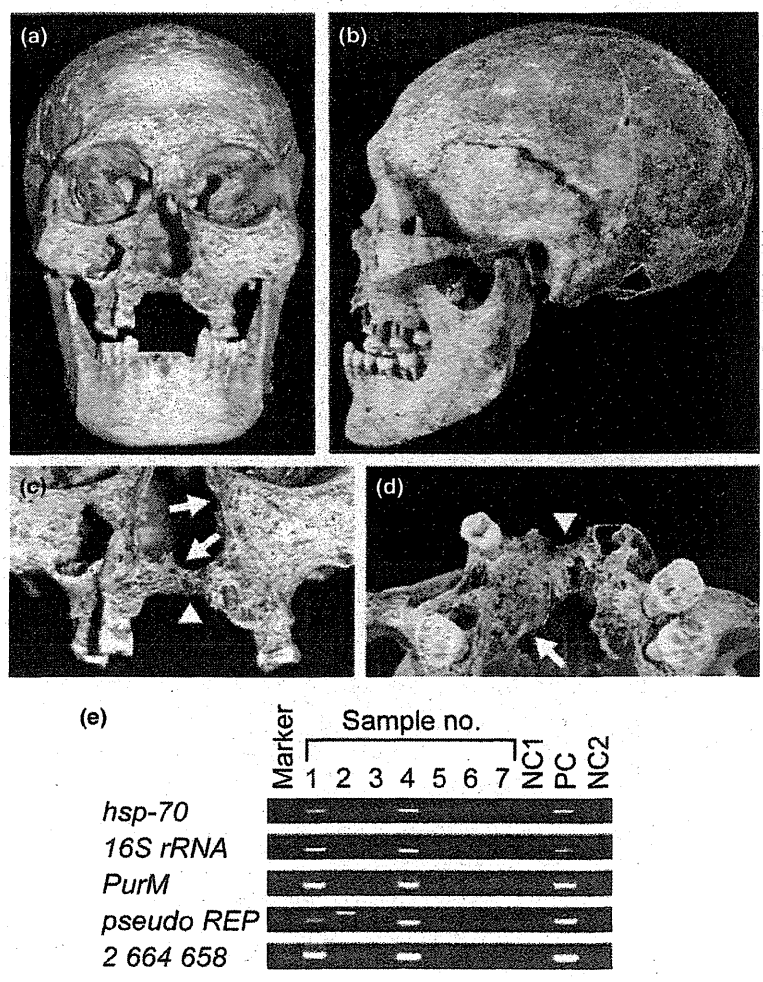

Figure 3. Skeletal lesions of leprosy and isolation of lesion-associated *Mycobacterium leprae* DNA.[59] Frontal view (a) and left side view (b) of archaeological skeletal remains showing erosive deformity of the nasal aperture and disappearance of the anterior nasal spine (arrows) and severe atrophy of the alveolar bone in the maxilla/palatal process with loss of anterior teeth (arrowheads) in panels (c) and (d). Polymerase chain reaction detection of *M. leprae* DNA from skeletal samples (samples 1–4). Samples 5–7 were taken from other skeletons found in the same cemetery, which had no leprous changes as a negative control. *M. leprae* DNA was detected in sample 1 (maxillary palate) and 4 (fibula) (e).

TREATMENTS

The implementation of MDT for leprosy treatment has been successful over the past three decades. The WHO has designed two easy-to-use blister pack medication kits for paucibacillary and multibacillary patients. The kits contain enough medication for 28 days and are supplied at no cost to registered patients. The treatment for paucibacillary patients include daily doses of 100 mg DDS and a monthly dose of 600 mg rifampicin (RFP) over a 6-month period. Multibacillary patients are administrated 100 mg DDS and 50 mg clofazimine (CLF) once a day in addition to monthly administration of 600 mg RFP and 300 mg CLF for 12 months. Treatment is usually automatically terminated at the end of the proscribed regimen because, in public health terms, it is reasonable to conclude that infectiousness is unlikely after starting MDT (Fig. 4).[69] Many countries, however, prefer longer treatments, especially for

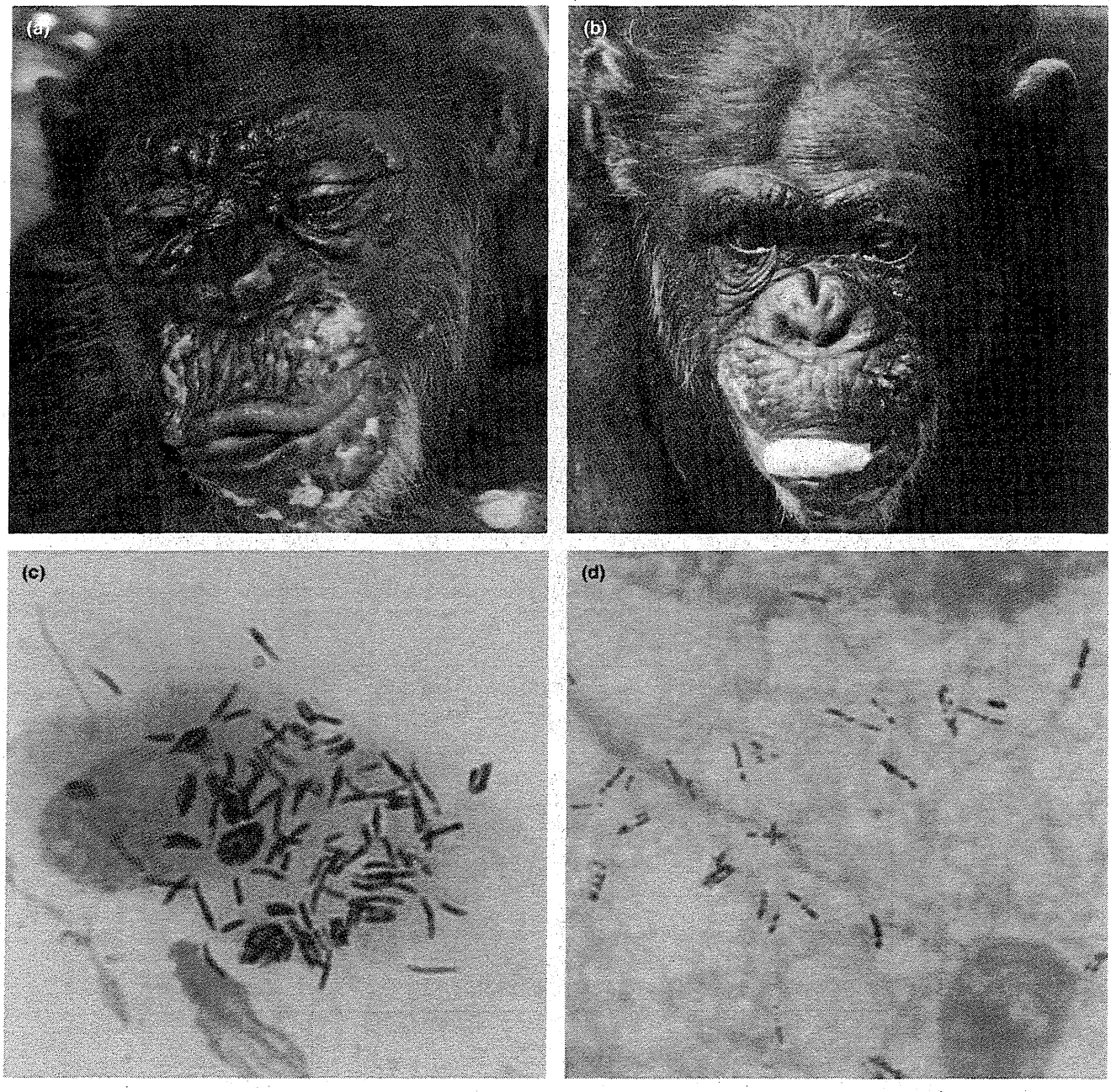

Figure 4. Female chimpanzee at leprosy diagnosis (a) and 3 months after the initiation of multidrug therapy (MDT), showing significant improvement of facial lesions (b).[30] Intact *Mycobacterium leprae* bacilli before treatment (c) fragmented and showed a granular staining pattern 6 months after MDT (d).