key role on stigma reduction in the community. Therefore, reasonable proportion of resources has been used for these activities. Priority population of IEC activities included community leaders and young people.

c. Strengthening case detection and management

- New case detection is always an important indicator of the programme. Good community awareness encouraged people with suspect signs and symptoms to go to the specialist for diagnosis confirmation. Other kinds of examinations are also carried out including contact examination, integrated examination, group examination...

In addition, SAPEL and LEC are also implemented in high endemic zones.

- All detected patients were treated with MDT free of charge. The duration of treatment for PB patients was 6 month with observed monthly dose of rifampicin and daily dose dapsone. For MB patient observed monthly dose of rifampicin and clofazimine was combined with daily dose of clofazimin and dapsone. The duration of treatment for MB patients used to be 24 months and then shortened to 12 months from 2005. The MDT storage was available at all communal health stations, even to all the village in the endemic zones.
- Handbooks were distributed to the patients, giving them useful guides to early detect the complications including, drug eruption, neuritis and other sign effects. All patients with these problems were referred to leprosy specialists for diagnosis.

d. Disabilities prevention and rehabilitation

Due to lack of financial resources, these activities were only under attention from 2000. Patients were taught to prevent themselves from deformities through handbooks and training courses. 80% of patients were provided with special shoes to prevent foot injury. 50% of the patients with eye closure problem were provided with special glasses. More than 1,000 patients received reconstructive surgery annually.

e. Conducting post-elimination surveillance project

A pilot post-elimination surveillance project based on protocol developed by WPRO has been operating in selected 40 provinces since 2002. The project has proven to be very effective for the leprosy control programme. Many training courses on leprosy field have been held for the health care workers at various levels. Provincial referral centers have been established, where many suspected cases have been to referred to confirm the diagnosis

f. Receiving national and international aids

The national leprosy control programme gained active support of national and international organizations, *i.e.* DFB, GLRA, NLR, AIFO, SMHF and Unilever Vietnam. Each year, national leprosy control programme received about 15 billions VND from the government including medicine, medical equipments and training fees.

3. Major achievements of the programme from 1995 - 2006

The significant achievements in reducing the burden of leprosy over twelve years were the result of success in implementing the mentioned solutions.

- Between 1995 and the end of 2006, about 100 millions participations were examined for leprosy detection
- Social stigma was removed, patients were treated at their home and they could work together with other community members. Their children can go

to school.

- WHO's elimination goal was reached at all provinces at the end of 2000.
- 19,429 new cases detected (of whom 12,099 were MB and 7,330 were PB) (Figure 5)
- Prevalence rate dropped from 0.70/10,000 population in 1995 to 0.07/10,000 population in 2006 (Figure 2)
- Child proportion among new cases reduced from 8.57% in 1995 to 5.56% in 2006 (Figure 6)
- Detection rate declined from 3.45/100,000 population in 1995 to 0.79/100,000 population in 2006 (Figure 3)
- Grade 2 disability rate among new cases decreased from 30.50% in 1995 to 17.27% in 2006 (Figure 4)
- Nearly 24,000 patients completed MDT with the completion rate was 99% (Figure 7)
- About 16,000 patients received rehabilitation activities

Year	No of new cases	Rate (1/100,000)
1995	2591	3.45
1996	2883	3.83
1997	2808	3.65
1998	2162	2.74
1999	1795	2.35
2000	1477	1.94
2001	1336	1.73
2002	1158	1.44
2003	940	1.18
2004	858	1.04
2005	746	0.90
2006	666	0.79

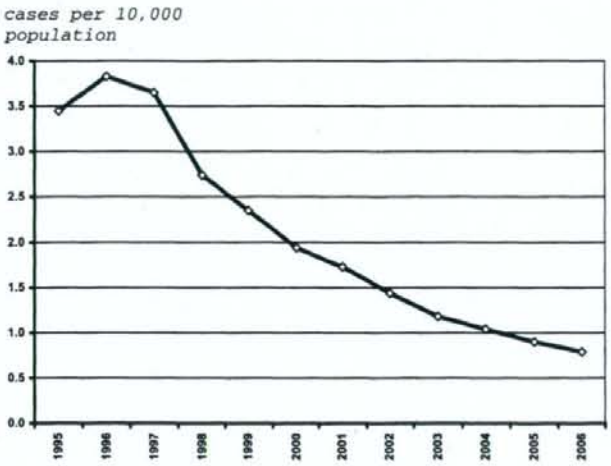

Figure 3. Detection rate of leprosy (1995-2006)

Year	No of new cases	Cases with grade II disability	Rate (%)
1995	2591	789	30.45
1996	2883	909	31.53
1997	2808	854	30.41
1998	2162	626	28.95
1999	1795	450	25.07
2000	1477	309	20.92
2001	1336	267	19.99
2002	1158	225	19.43
2003	949	179	18.86
2004	858	145	16.90
2005	746	121	16.22
2006	666	115	17.27

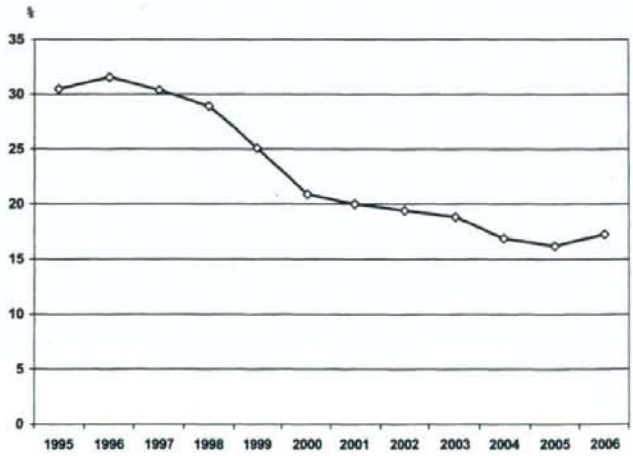

Figure 4. Grade II disability proportion among new cases (1995-2006)

4. Reduced stigma of leprosy in Vietnam

Leprosy has affected people in Vietnam at least hundreds of years ago. There were scattered writings on leprosy and leprosy affected patients in old documents. In the past, stigma towards leprosy patients and their families had adversely affected their quality of life due to its impacts on their mobility, interpersonal relationships, marriage, job finding, leisure and other social activities. All of these occurred in the community despite the fact that no segregation or discrimination laws has been promulgated to date.

At that time, people believed that leprosy had bad origin and was the punishment for sins the patients committed in the previous incarnation. In some communities, there is a belief that leprosy was hereditary so leprosy patients were forced to be infertilized.

Since National leprosy control programme was established, many activities have been done to re-

Year	No of new cases	No of MB cases	Rate (%)
1995	2591	1690	65.23
1996	2883	1807	62.68
1997	2808	1798	64.03
1998	2162	1278	59.11
1999	1795	1071	59.67
2000	1477	905	61.27
2001	1336	822	61.53
2002	1158	715	61.74
2003	949	590	62.17
2004	858	560	65.27
2005	746	452	60.59
2006	666	411	61.71

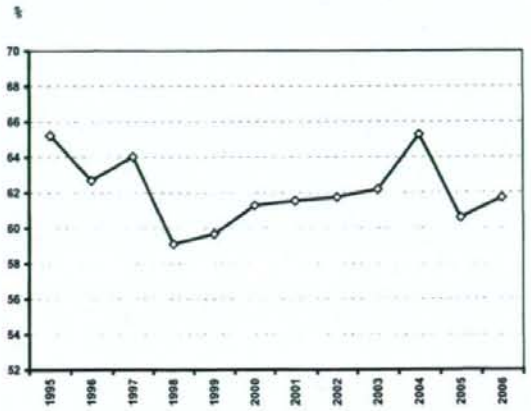

Figure 5. MB proportion among new cases (1995-2006)

Year	No of new cases	Caes with age < 15	Rate (%)
1995	2591	222	8.57
1996	2883	211	7.32
1997	2808	159	5.66
1998	2162	162	7.49
1999	1795	124	6.91
2000	1477	105	7.11
2001	1336	77	5.76
2002	1158	65	5.61
2003	949	52	5.48
2004	858	47	5.48
2005	746	47	6.30
2006	666	37	5.56

Figure 6. Children proportion among new cases (1995-2006)

duce the stigma in the community. The implementation of MDT, which effectively treated leprosy patients, itself eased the sigma burden by reducing visible deformity rate. The old words indicating leprosy such as "*cùi*" in the south part or "*hủi*" in the north part of the country have no longer been used because they imply something evil, degrading or immoral. Instead, the more scientific and natural term "*phong*" has been used to call the disease. In addition, higher knowledge of the curability and difficult-to-contamination of leprosy has been popularized and lead to more positive attitudes towards person affected with leprosy. The main features of leprosy are taught in the elementary school in the endemic areas.

Misconceptions about the causes of leprosy, that could have perpetuated stigma, were also prevalent among health care workers in the past. But the misconceptions have been gradually reduced due to the retraining courses held by the National Programme which provided health care workers with more accurate knowledge of leprosy. Moreover, the antileprosy work, since last decade, has been integrated into the general health care system, so that people recognized that leprosy is actually an infectious disease and deformed persons by leprosy are just like other handicapped person.

As a result of intensive activities, leprosy patients are now hardly affected by stigma from the society. Patients are treated free of charge with MDT at their own home and they can have a job suitable to their health. Marriage is accepted for leprosy patients and many patients have children after diagnosis. Public and private schools allow children born to leprosy patients to attend, and these children can study with other children in the community.

5. Post-elimination challenges

Even WHO's elimination goal was reached at all provinces at the end of 2000, relatively high case detection rate was still found in some area particularly in the central highland and some southern provinces. In 2006, there were 20 provinces reporting detection rate more than 1/100,000 population. The patients were not distributed equally but there were many pockets of patients that need more attention from the programme.

At the end of 2006, only 32 provinces reached

Year	PB	MB	Total
1995	1011	2449	3460
1996	1046	1785	2831
1997	981	1688	2669
1998	972	2257	3229
1999	839	2221	3060
2000	610	1210	1820
2001	568	910	1478
2002	515	847	1362
2003	331	711	1042
2004	555	654	1209
2005	534	409	943
2006	401	367	768

Figure 7. Number of patients complete MDT (1995-2006)

the Vietnam's 4 elimination criteria and 5 provinces still had prevalence rate more than 0.2/10,000 population. MB and child proportion were high, 61.71% and 5.56% respectively, indicating high infectiveness.

In the areas where prevalence rate is low, the health staffs in charge of leprosy have to do many other activities with different topics and there are few chances for them to see the lesions of active leprosy. As the result, their knowledge and practices regarding leprosy may be lost gradually.

6. Post-elimination activities

Based on the effectiveness of the post-elimination leprosy surveillance, the rest 24 provinces need to be covered by activities of the project. In the next period, the objects of the project are:

- sustaining the network of leprosy control programme despite the fact that low prevalence and detection rates in selected provinces
- conducting retraining courses on leprosy for the health staff at provincial, district and communal levels
- detecting all hidden cases of leprosy in the community
- sustaining and strengthening the awareness of leprosy in the community with IEC activities

7. Future requiremental aids

In the future, community directed activities must be carried out to maintain the achievements. In addition, high technologies on leprology are to be developed in central and regional institutes such as sequencing to detect drug resistance of mycobacteria; serology and other tests to make prognosis in high risk groups. Aids from developed countries are the important part of this plan.

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RESEARCH LETTER

Expression of adipose differentiation-related protein (ADRP) and perilipin in macrophages infected with *Mycobacterium leprae*

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Mycobacterium leprae; leprosy; lipid; adipose differentiation-related protein; perilipin.

Abstract

Mycobacterium leprae survives and replicates within a lipid droplet stored in the enlarged phagosome of histiocytes, a typical feature of lepromatous leprosy that is thought to be an important nutrient source for the bacillus. However, the underlying mechanisms by which lipids accumulate within phagosomes remain unclear. Recently, it was revealed that the lipid droplet-associated proteins, including ADRP and perilipin, play essential roles in lipid accumulation in adipocytes or macrophages. Therefore, we attempted to examine the role of these proteins in leprosy pathogenesis. ADRP and perilipin localized to the phagosomal membrane, which contains *M. leprae* in skin biopsy specimens of lepromatous leprosy. ADRP expression was transiently increased after phagocytosis in THP-1 cells. However, high levels of ADRP expression persisted only when live *M. leprae*, but not dead bacilli or latex beads, was added. Furthermore, although peptidoglycan, a Toll-like receptor 2 ligand, suppressed the expression levels of ADRP and perilipin, *M. leprae* infection inhibited this suppression. These results suggest that live *M. leprae* has the ability to actively induce and support ADRP/perilipin expression to facilitate the accumulation of lipids within the phagosome and to further maintain a suitable environment for the intracellular survival within the macrophage.

Introduction

Leprosy, a chronic infectious disease caused by *Mycobacterium leprae*, shows a broad spectrum of clinical manifestation. Lepromatous leprosy is characterized by widespread skin lesions consisting of unrestricted multiplication of bacilli inside foamy histiocytes due to an impaired cellular immune response. In these lesions, *M. leprae* lives and replicates in a foamy or an enlarged phagosome within macrophages that are filled with lipids.

However, it is unclear how such a large amount of lipids is recruited and accumulated in phagosomes containing *M. leprae*. It is thought that *M. leprae* survives by utilizing the lipids and fatty acids as carbon source in the granuloma environment, where the oxygen tension gradient is relatively low (Chan *et al.*, 1989). Therefore, it is important to understand the mechanisms by which lipid droplets accumulate within the phagosome in order to better understand the strategy that *M. leprae* uses to survive within host cells.

Recent studies have highlighted the important role of proteins that mediate lipid accumulation in cells. In animal cells, these include the structurally related members of the PAT protein family, which is named after perilipin, adipophilin/adipose differentiation-related protein (ADRP), and the tail-interacting protein of 47 kDa (TIP47) (Greenberg *et al.*, 1993; Blanchette-Mackie *et al.*, 1995; Servetnick *et al.*, 1995; Brasaemle *et al.*, 1997a, b; Wolins *et al.*, 2001; Miura *et al.*, 2002). ADRP is a ubiquitously expressed PAT family

protein that serves as a scaffolding during lipid droplet formation. The protein has fatty acid-binding properties and stimulates fatty acid uptake in cells (Gao *et al.*, 2000). Overexpression of ADRP increased triglyceride accumulation, and knockdown of ADRP by a specific small interfering RNA decreased the pool of cytosolic lipid droplets (Magnusson *et al.*, 2006). ADRP expression has been suggested for use as a sensitive marker of lipid loading in human blood monocytes and in human monocyte-derived macrophages incubated with oxidized low-density lipoproteins (LDL) (Llorente-Cortes *et al.*, 2007). In contrast, perilipin was originally described as a lipid droplet-associated protein expressed only in adipocytes (Serlachius & Andersson, 2004), but it has been identified recently in other tissues including the vascular wall, where expression was demonstrated in macrophages and smooth muscle cells (Forcheron *et al.*, 2005). Studies on perilipin null mice suggest that perilipin shields lipid droplets from hormone-sensitive lipase activity under basal conditions and is necessary for cyclic adenosine monophosphate-stimulated triglyceride lipolysis (Sztalryd *et al.*, 2003; Miyoshi *et al.*, 2006). Perilipin has also been found on the surface of lipid droplets in lipid-loaded human-cultured THP-1 monocytes (Persson *et al.*, 2007), and the protein is expressed in primary human macrophages incubated with acetylated LDL. Perilipin expression increased over time in cell culture when human monocytes spontaneously differentiated into macrophages (Persson *et al.*, 2007). Furthermore, perilipin content has been correlated with lipid content in foam cells (Larigauderie *et al.*, 2006).

To date, it is not known whether ADRP/perilipin play roles in lipid accumulation in lepromatous leprosy, a typical example of intracellular parasitization of bacteria. In this study, we examine the expression and localization pattern of ADRP/perilipin both *in vivo* and *in vitro* and explore the impact of *M. leprae* on these cellular activities.

Materials and methods

Acid-fast staining and immunohistochemistry

Archived formalin-fixed, paraffin-embedded tissue sections were subjected to immunohistochemical staining as described previously (Suzuki *et al.*, 1998a, 2006b). Briefly, deparaffinized sections were incubated with anti-ADRP antibody (PROGEN Biotechnik GmbH, Heidelberg, Germany) diluted to 1 : 200 or anti-perilipin antibody (Affinity BioReagents, Golden, CO) diluted to 1 : 100 for 1 h at room temperature. Slides were washed with Dulbecco's phosphate-buffered saline containing 0.1% polyoxyethylene sorbitan monolaurate (Tween 20). The peroxidase-labeled streptavidin–biotin method using the LSAB2 Kit (DAKO, Carpinteria, CA) and 3,3-diaminobenzidine tetrahydrochloride (DAB) was used according to the manufacturer's protocol (Suzuki *et al.*, 1998a, b, 2006b). Sections were then stained with carbol fuchsin and counterstained with methylene blue to visualize acid-fast mycobacteria. Archived formalin-fixed, paraffin-embedded tissues were used according to the guidelines approved by the National Institute of Infectious Diseases (Tokyo, Japan).

Cell culture and infection with *M. leprae*

THP-1, a human promonocytic cell line, was obtained from the American Type Culture Collection (ATCC; Manassas, VA). Cells were cultured in 10 cm tissue culture dishes in RPMI medium supplemented with 10% charcoal-treated fetal bovine serum, 2% nonessential amino acids, and 50 mg mL^{-1} penicillin/streptomycin at 37 °C in 5% CO_2. *Mycobacterium leprae* was prepared from the footpads of nude mice as described previously (Suzuki *et al.*, 2006a, b). Live or heat-killed (80 °C, 30 min) bacilli (3×10^7) or latex beads (Fluoresbrite microspheres; Technochemical, Tokyo, Japan) were added to 3×10^6 cells, multiplicity of infection (MOI) 10. Cells were further cultured for RNA and protein purification.

RNA preparation, reverse transcriptase (RT)-PCR and quantitative real-time PCR

RNA was prepared from cultured cells using the RNeasy Mini Kit (Qiagen Inc., Valencia, CA) as described previously (Suzuki *et al.*, 1999a, b, 2006a). RNA preparation from skin smear samples was performed as follows: Stainless-steel blades (Feather Safety Razor Co. Ltd, Osaka, Japan) used to obtain slit-skin smear specimens were rinsed in 1 mL of sterile 70% ethanol and centrifuged at 20 000 *g* for 1 min at 4 °C. RNA was isolated from pellets with an RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany), using the same protocol as that used for cultured cells. RNA was eluted in 20 μL of elution buffer and treated with 0.1 U μL^{-1} of DNase I (TaKaRa, Kyoto, Japan) at 37 °C for 1 h in order to degrade any contaminating genomic DNA. RNA concentration and purity were assessed using a Genequant Pro Spectrophotometer (GE Healthcare UK Ltd, Buckinghamshire, UK). Total RNA from each sample was reverse-transcribed to cDNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, CA) as described by Suzuki & Kohn (2006) and Suzuki *et al.* (2006a). The following primers were used to amplify cDNA: 5′-TGTGGAGAAGAC CAAGTCTGTG-3′ (ADRP forward) and 5′-GCTTCTGAAC CAGATCAAATCC-3′ (ADRP reverse); 5′-GCTCTGATTCT ATGGCTTGGTT-3′ (perilipin forward) and 5′-TGTGTCA AAACCTTCTGTCTGG-3′ (perilipin reverse); and 5′- AGC CATGTACGTAGCCATCC-3′ (actin forward) and 5′-TGTG GTGGTGAAGCTGTAGC-3′ (actin reverse). Touchdown PCR was performed using a Thermal Cycler Dice (Takara)

as described previously (Suzuki *et al.*, 1998a, b, 2006a). The products were analyzed by 2% agarose gel electrophoresis. Skin smear samples were taken after obtaining written informed consent.

Real-time PCR was carried out in a solution containing 10 μL of SYBR Green PCR Master Mix (Applied Biosystems), 50 nM of each primer, and cDNA template. The same primers that were used for RT-PCR analysis were utilized. Samples were analyzed using an ABI Prism 7000 analyzer (Applied Biosystems) with an initial step of 2 min at 50 °C and 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Data were analyzed using ABI PRISM 7000 SDS Software Version 1.1 (Applied Biosystems). All samples were amplified in triplicate from the same RNA preparation, and the experiment was repeated three times.

Protein preparation and Western blot analysis

Cellular protein was extracted and analyzed as described previously (Suzuki *et al.*, 1999a, b, 2002). 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 Diagnostics, Basel, Switzerland) for 1 h. After centrifugation, the supernatant was transferred and 20 μg of protein was used for Western blotting. Samples were heated in sodium dodecyl sulfate sample loading buffer at 95 °C for 5 min and loaded on a polyacrylamide gel. After electrophoresis, proteins were transferred to a polyvinylidene difluoride membrane using a semi-dry blotting apparatus (Bio-Rad, Hercules, CA). The membrane was washed with PBST (PBS with 0.1% Tween 20), placed in blocking buffer (PBST containing 5% nonfat milk) overnight, and then incubated with anti-ADRP (1 : 1000) or anti-perilipin (1 : 1000) antibody. After washing with PBST, the membrane was incubated for 1 h with biotinylated donkey anti-rabbit antibody (Amersham Biosciences, Piscataway, NJ) and streptavidin–HRP (Amersham Bioscience), according to the manufacturer's protocol, and developed using ECL Plus reagent (Amersham Biosciences).

Transient transfection and luciferase assay

A luciferase reporter plasmid, p5 × NF-κB-luc, was purchased from Stratagene (La Jolla, CA). Transient transfection was conducted using FuGene 6 (Roche Diagnostics, Indianapolis, IN) according to the manufacturer's protocol (Suzuki *et al.*, 1998a, b, 1999a, b). THP-1 cells were incubated for 36 h after transfection, after which peptidoglycan (2 μg mL^{-1} final concentration) or *M. leprae* (MOI 10) was added. Luciferase activity was measured using the Luciferase Reporter Assay System (Promega, Madison, WI) according to the manufacturer's protocol (Suzuki *et al.*, 1998a, b, 1999a, b).

Other reagents

All other reagents were purchased from Sigma Aldrich (Saint Louis, MO).

Statistical analysis

Results are expressed as mean ± SE. Student's *t*-test was used for statistical analysis. P values < 0.05 were regarded as statistically significant.

Results

ADRP and perilipin are expressed in skin lesions of lepromatous leprosy

ADRP and perilipin expression was assayed in skin biopsy specimens taken from patients with lepromatous leprosy. Immunohistochemical staining was used to visualize ADRP and perilipin, and *M. leprae* was demonstrated using acid-fast staining on the same section (double staining). The immunoreactivity of ADRP and perilipin was observed in the majority of foamy histiocytes that contain acid-fast bacilli (i.e. *M. leprae*) (Fig. 1a and b, respectively). At a higher magnification, both were clearly observed on the membranes of phagosomes containing *M. leprae* (Fig. 1c and d). The staining was also observed in old lesions of lepromatous leprosy where bacilli were primarily degenerative or not visible (Fig. 1e and f), which is similar to the case of CORO1A localization (Suzuki *et al.*, 2006b). However, expression of ADRP and perilipin was not detected in the granulomatous lesions of tuberculoid leprosy where *M. leprae* is not usually detected (Fig. 1g and h). RT-PCR of the skin smear specimens confirmed the presence of ADRP/perilipin mRNA in all of the samples tested, although the expression levels were variable among samples (Fig. 2). These results indicate that ADRP/perilipin is expressed in the lesions of lepromatous leprosy and localizes to the phagosomal membrane of histiocytes that contain *M. leprae*.

ADRP/perilipin expression is induced by *M. leprae* infection in THP-1 cells

We next examined ADRP/perilipin expression in human THP-1 cells following *M. leprae* infection. RT-PCR analysis revealed a rapid increase in ADRP mRNA 1 h after infection and in perilipin mRNA 6 h after infection, which was maintained for 12 h postinfection (Fig. 3a). The increase in mRNA levels was more prominent when a larger number of bacilli (MOI 100) were used (data not shown). Western blot analysis demonstrated a significant increase in ADRP protein as early as 3–6 h after infection and within 9–12 h for perilipin protein (Fig. 3b). Immunocytochemistry showed that both ADRP and perilipin localized to the phagosomal membrane of THP-1 cells containing *M. leprae* (Fig. 3c and d,

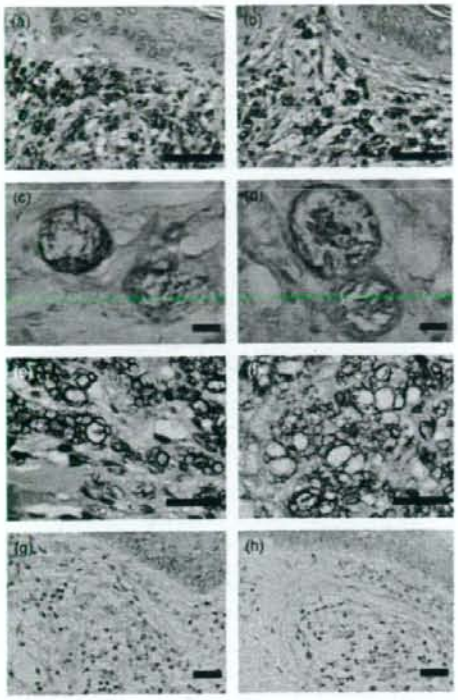

Fig. 1. Localization of ADRP and perilipin in skin lesions of lepromatous leprosy. Formalin-fixed, paraffin-embedded tissue sections from fresh (a, b, c, and d) and old (e and f) lesions from lepromatous leprosy and from tuberculoid leprosy (g and h) were immunostained for ADRP and perilipin (brown coloration), followed by acid-fast staining (pink coloration) and methylene blue or hematoxylin counterstaining. Photomicrographs of ADRP (a, c, e, and g) and perilipin (b, d, f, and h) immunostaining are shown. Original magnification: × 200 (a, b, g, and h; scale bar = 25 μm); × 1000 (c and d; scale bar = 5 μm); × 400 (e and f; scale bar = 25 μm).

respectively). Immunofluorescence staining confirmed the results (data not shown).

To clarify whether the observed increase of ADRP/perilipin is specific for *M. leprae* or is instead nonspecific for phagocytosis, we compared the effect of introducing live *M. leprae*, heat-killed *M. leprae*, or latex beads. In all three cases, a similar increase in ADRP/perilipin mRNA expression was observed by RT-PCR (Fig. 4a) and quantitative real-time PCR in 6 h (Fig. 4b and c). Interestingly, however, ADRP expression remained at a high level for 72 h only in cultures to which live *M. leprae* was added (Fig. 4a and b). Perilipin mRNA reverted to original levels in 72 h in all the cases (Fig. 4a and c). Correspondingly, the transient expression of ADRP protein levels in 6 h and its prolonged expression following live *M. leprae* infection were confirmed

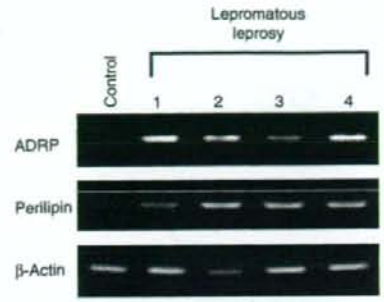

Fig. 2. Detection of ADRP and perilipin mRNA in skin smear samples. RNA was isolated from skin smear specimens taken from four patients with lepromatous leprosy. After treatment with DNase, RT-PCR was performed. The control sample was obtained from skin granuloma where mycobacteria were not found.

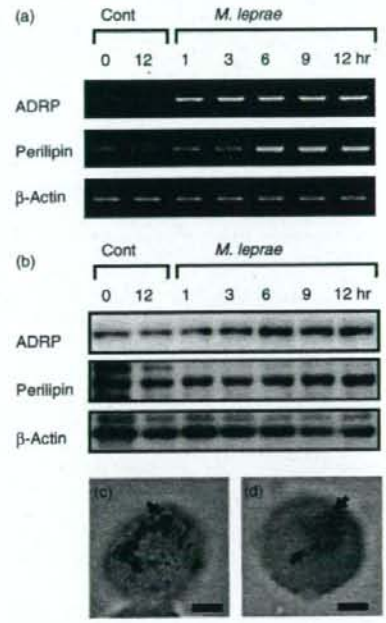

Fig. 3. Induction of ADRP and perilipin expression by *Mycobacterium leprae* infection of THP-1 cells. THP-1 cells (3×10^6) were cultured in a six-well plate and infected with 3×10^7 cells of *M. leprae*. After incubating for the indicated time, total RNA and total cellular protein were purified and RT-PCR analysis (a) and Western blot analysis (b) were performed. THP-1 cells grown on glass cover slips were infected with *M. leprae* and subjected to ADRP (c) and perilipin (d) immunocytochemistry. Arrows indicate a positive signal around phagosomes. Original magnification: × 1000. Scale bar = 3 μm.

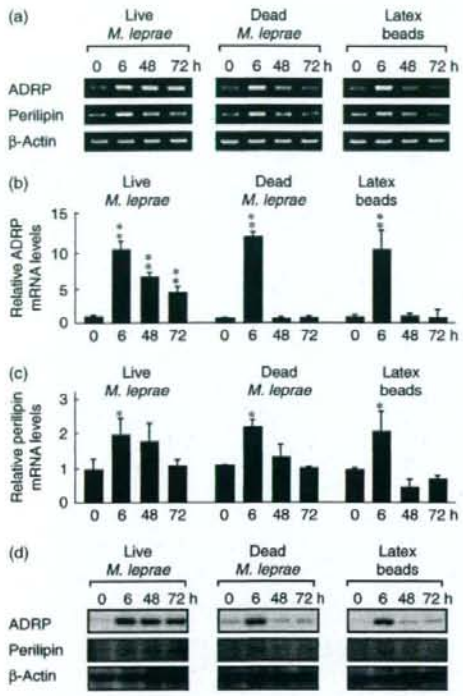

Fig. 4. Only live *Mycobacterium leprae* induces expression of ADRP. THP-1 cells (3×10^6) were cultured in a six-well plate and infected with 3×10^7 cells of live *M. leprae*, heat-killed (80 °C for 30 min) *M. leprae* or latex beads. After incubating for the indicated time, total RNA was purified and RT-PCR analysis (a) and quantitative real-time PCR (b and c) was performed. The results from real-time PCR were normalized with actin expression and expressed as a relative value against 0 h. Similarly, cellular protein was purified and Western blot analyses of ADRP and perilipin were performed (d). The graph shows the mean ± SD. One asterisk indicates a value of $P < 0.05$; two asterisks indicate a value of $P < 0.001$.

by Western blot analysis (Fig. 4d). The increase in perilipin protein levels was limited, as were the changes in mRNA levels. Because the phagosome is formed within a few hours after phagocytosis of mycobacteria (Ferrari *et al.*, 1999; Suzuki *et al.*, 2006b), these results suggest that transient expression of ADRP/perilipin is induced by phagocytosis; however, expression of ADRP is sustained by an unknown component(s) derived from live *M. leprae*.

Mycobacterium leprae infection reverses the effect of peptidoglycan on suppression of ADRP mRNA expression

It is well known that cell wall lipoproteins of mycobacteria stimulate Toll-like receptor 2 (TLR2) and activate a downstream signaling cascade (Jones *et al.*, 2001). Therefore, we first attempted to evaluate the effect of TLR-mediated signaling in *M. leprae*-infected macrophages. Phorbol myristate acetate (PMA)-treated THP-1 cells were used as a model of activated macrophages in which to evaluate the effect of peptidoglycan and *M. leprae*. The amount of peptidoglycan ($2\,\mu g\,mL^{-1}$) and *M. leprae* (MOI 10) used similarly induced NF-κB-dependent promoter activation (Fig. 5a), suggesting that both have a similar ability to stimulate TLR2. PMA strongly induced ADRP mRNA levels as reported (Wei *et al.*, 2005), but the effect was weak on perilipin mRNA levels (Fig. 5b and c). Peptidoglycan strongly reduced the ADRP mRNA levels and weakly reduced perilipin expression (Fig. 5d). Interestingly, *M. leprae* infection did not affect ADRP expression levels at all, and modulated perilipin expression only weakly (Fig. 5e). We then examined the effect of *M. leprae* on the ADRP/perilipin levels that were reduced by peptidoglycan. The addition of *M. leprae* reversed the suppressive effect of peptidoglycan, allowing continued expression of high levels of ADRP (Fig. 5f). The results of quantitative real-time PCR analysis of ADRP mRNA levels confirm these results (Fig. 5g). Perilipin mRNA levels were not significantly affected by *M. leprae* in PMA-activated THP-1 cells (Fig. 5e and f; real-time PCR data not shown). These results suggest that *M. leprae* infection inhibits TLR2-mediated suppression of ADRP expression.

Discussion

In this study, we demonstrated that ADRP/perilipin localizes to the phagosomal membrane of histiocytes, which contains numerous bacilli, in skin lesions of lepromatous leprosy. In addition, we showed that *M. leprae* infection increases expression of ADRP/perilipin mRNA and protein in THP-1 cells. These results suggest that *M. leprae* regulates ADRP/perilipin expression for the accumulation of lipid droplets that will be utilized as a nutrient for intracellular survival.

In fact, there is evidence that pathogenic mycobacteria primarily use fatty acids rather than carbohydrates as carbon substrates during infection. Respiration of *Mycobacterium tuberculosis* grown in mouse lungs is strongly stimulated by fatty acids but is unresponsive to carbohydrates (Zahoor *et al.*, 2005). Several glycolytic enzymes are apparently dispensable for growth and persistence of *M. tuberculosis* in mice (Mathur *et al.*, 2005), and the terminal step in glycolysis is blocked in the closely related zoonotic pathogen *Mycobacterium bovis* as a result of a mutation in *pykA*, which encodes pyruvate kinase (Keating *et al.*, 2005). Furthermore, persistence of *M. tuberculosis* in mice is facilitated by isocitrate lyase, an enzyme essential for the metabolism of fatty acids (Gould *et al.*, 2006). Therefore, it would be

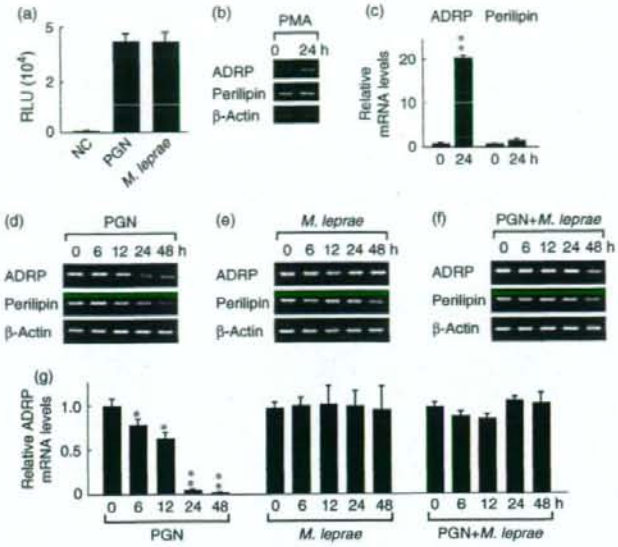

Fig. 5. *Mycobacterium leprae* inhibits the ability of peptidoglycan to suppress the expression of ADRP. TLR2 activation in THP-1 cells was assessed with a luciferase assay using an NF-κB-dependent reporter gene (a). THP-1 cells (3×10^6) were cultured in a six-well plate and treated with PMA at a final concentration of 20 ng mL^{-1} for 24 h. mRNA expression of ADRP and perilipin was assessed using RT-PCR (b) and quantitative real-time PCR (c). The results from real-time PCR were normalized with actin expression and reported as a relative value against 0 h. PMA-stimulated cells were treated with peptidoglycan (2 μg mL^{-1}) (d), *M. leprae* (MOI 10) (e), or both (f). After incubating for the indicated time, total RNA was purified and RT-PCR analysis was performed. Changes in ADRP mRNA levels were further evaluated by quantitative real-time PCR (g). The graph shows the mean ± SD. *, $P < 0.05$; **, $P < 0.001$.

plausible to speculate that *M. leprae* also utilizes fatty acids as carbon substrates within host cells.

Only live cells of *M. leprae* could sustain prolonged expression of ADRP/perilipin, while transient expression was induced by dead bacilli or latex beads. This situation is quite similar to the accumulation of CORO1A, also known as tryptophan aspartate-containing coat protein, on the phagosomal membrane, which results in inhibition of lysosomal fusion and accounts for the survival of bacilli (Ferrari *et al.*, 1999). CORO1A accumulates in the phagosomal membrane that contains *M. leprae* in lepromatous leprosy (Suzuki *et al.*, 2006b). Furthermore, only live, but not heat-killed, *M. bovis* Bacillus Calmette–Guérin could maintain CORO1A expression and localization on the phagosome (Ferrari *et al.*, 1999). Therefore, *M. leprae* might actively recruit ADRP/perilipin, as well as CORO1A, to the phagosomal membrane to create an appropriate and favorable environment within the phagosome. Although it is difficult to identify responsible component(s) maintaining ADRP expression because of the lack of an *in vitro* cultivation method of *M. leprae*, our results potentially suggest that *M. leprae* have an ability to stimulate ADRP expression as well as CORO1A expression. It appears that high expression levels of ADRP and perilipin were maintained in the clinical specimens (Figs 1 and 2), while expression decreased after several hours in cultured THP-1 cells (Fig. 4). This may be a reflection of a current limitation of leprosy research – *in vitro* cultivation of *M. leprae*, even in cultured cells, is not possible. Therefore, continuous stimulation of live *M. leprae* cannot be carried out for a long period of time *in vitro*. Whether other pathogenic and nonpathogenic mycobacteria have similar effects on ADRP/perilipin induction is an interesting issue for future study.

The changes in mRNA expression pattern and protein levels differ between ADRP and perilipin. It has been shown in adipocytes that the proteins that coat lipid droplets, which are determined by multiple factors, change during lipid droplet biogenesis (Ducharme & Bickel, 2008). Although the precise molecular mechanisms that regulate transcription of ADRP and perilipin in macrophages have not been resolved, our results suggest the differential roles of two PAT proteins in *M. leprae*-infected macrophages.

TLR2 also localizes to phagosomal membranes that contain *M. leprae* (Suzuki *et al.*, 2006b). In the present study, peptidoglycan, a TLR2 ligand, suppressed ADRP/perilipin expression in macrophages. It is known that bacterial cell wall components bind TLR2 and stimulate downstream signaling cascades. This signal activates the expression of proinflammatory cytokines, chemokines and type I interferons in order to launch innate immunity as a first line of defense against infection (Underhill *et al.*, 1999). Thus, based on the present finding, we speculate that activation of innate immunity results in suppression of ADRP/perilipin, which in turn reduces lipid accumulation within the infected macrophage and accounts for the nutritional diminution for the intracellular pathogen.

We also showed that *M. leprae* inhibits TLR2-mediated suppression of ADRP expression. Because both peptidoglycan

and *M. leprae* induce similar activation of NF-κB under the conditions used, the underlying molecular mechanism by which *M. leprae* exerts such an opposite effect is unknown. It is speculated that *M. leprae* activates a hitherto unrecognized TLR-independent pathway that results in inhibition of TLR-mediated ADRP/perilipin suppression. Such a function would further contribute to the creation of a lipid-rich environment that is favorable for survival of the pathogen.

In conclusion, we have identified a mechanism that may contribute to the lipid accumulation observed in the foamy histiocytes of lepromatous leprosy. *Mycobacterium leprae* induces and actively sustains ADRP expression. Further studies determining the detailed mechanisms by which lipids accumulate in the *M. leprae*-infected phagosome will provide a better understanding of leprosy pathogenesis. It will also provide insights that may lead to the development of a new therapeutic method that inhibits the expression and/or the localization of ADRP and perilipin.

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ニューキノロン系抗菌薬の構造式と抗らい菌活性の相関

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らい菌に対し強い殺菌作用を示すニューキノロン系抗菌薬（NQ）は、多剤耐性らい菌に対する治療薬として重要である。NQ などの化学療法薬は *in vitro* 活性がいくら強くとも血中半減期や組織移行性などの体内動態が劣るならば、強い *in vivo* 活性は期待できない。今回 Buddemeyer 法とヌードマウス足蹠法を用いてニューキノロン系抗菌薬の構造式と抗らい菌活性の相関を検討した。

実験結果からキノロン母核の 1 位にシクロプロピル基、3 位にカルボキシル基、4 位にオキシ基、5 位にアミノ基または水素基、6 位にフッ素基、7 位に 5 員環または 6 員環の塩基性環状アミン、8 位にフッ素基、塩素基またはメトキシ基に置換した NQ が、抗らい菌活性を最も強めることが示唆された。

はじめに

ハンセン病は、多剤併用療法の普及により有病率は低下したが、世界では今なお約 26 万人[1]の新患発生があるばかりか、耐性菌増加の問題も生じている。さらに少菌型で 6 ヵ月、多菌型で 1 年以上の長い治療期間を要する。rifampicin（RFP）を除くと唯一らい菌に対し殺菌作用を持つニューキノロン系抗菌薬（NQ）は多剤耐性菌の治療薬として重要で、すでに一部の NQ がハンセン病の治療に用いられている。ハンセン病は、らい菌により末梢神経と皮膚が侵される慢性感染症であることから NQ は *in vitro* 活性がいくら強くとも血中半減期や組織移行性、代謝安定性などの体内動態が劣るならば、強い *in vivo* 抗らい菌活性は期待できない。

ofloxacin（OFLX、第一三共）[2]は、キノロン骨格にオキサンジン環を有することを特徴とする三環系 NQ で、血中半減期は 4.5 時間（空腹時 200mg 単回経口投与）で、光毒性など副作用が少ない。levofloxacin（LVFX、第一三共）[3]は、ラセミ体である OFLX の一方の光学活性体 S-(—) の NQ で、血中半減期は 5.12 時間（空腹時 100mg 単回経口投与）で、光毒性など副作用が少ない。sparfloxacin（SPFX、大日本住友製薬）[4, 5]は、血中半減期が 15.8 時間（空腹時 100mg 単回経口投与）と長く、優れた組織移行性と強い抗菌力を持つ NQ である。sitafloxacin（STFX、第一三共）[6]は、血中半減期 5 時間（空腹時 100mg 単回経口投与）、強い抗菌力、特に MRSA や PRSP に強い活性を示すとともに、痙攣誘発などの副作用を軽減した NQ である。gatifloxacin（GFLX、杏林製薬）[7, 8]は、血中半減期 7.1 時間（空腹時 200mg 単回経口投与）、強い抗菌力と光毒性を軽減した NQ である。WQ-

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3402（湧永製薬）[9]は、血中半減期が2.7時間（10mg/kg, dogs, 単回経口投与）で、CPFX耐性菌やMRSAに対し強い抗菌活性を示すNQである。moxifloxacin（MFLX、バイエル薬品）[10]は、血中半減期が15.3時間（空腹時200mg単回経口投与）と長く、強い抗菌力、耐性菌出現抑制作用が強く、薬物相互作用と光線過敏症などに対する安全性を重視して開発されたNQである。garenoxacin（GRNX、富山化学工業）[11]は、既存NQと異なった構造式を有し、血中半減期が12.6時間（空腹時200mg単回経口投与）と長く、MRSAやVREなどの薬剤耐性菌に対し強い抗菌活性を示し、光線過敏症や薬物相互作用などNQ中最も副作用が少なく小児や高齢者への適用が期待されるNQである。

これら薬理学的特長を持つ8種類のNQをBuddemeyer法とヌードマウス足蹠法の実験結果から、NQの構造式と抗らい菌活性の相関を検討した。

材料と方法

1）らい菌：らい菌（Thai-53株）を接種後11ヶ月目のヌードマウス（BALB/c）の後肢足蹠より集菌・精製[12]し、Shepard法[13]により菌数計算後所定の濃度に希釈しBuddemeyer法とヌードマウス足蹠法に用いた。

2）動物：ヌードマウス（BALB/c-*nu/nu*、雌、5週令）は、日本クレアから購入し、ビニールアイソレータ（三基科学工芸）の中で、滅菌したマウス用耐圧固形飼料（MB-6E、船橋農場）で飼育した。

3）抗菌薬：OFLX, LVFX, STFX（第一三共）、SPFX（大日本住友製薬）、GFLX（杏林製薬）、WQ-3402（湧永製薬）、MFLX（バイエル薬品）、GRNX（富山化学工業）、は各製薬会社から原末の提供を受けた。

上記供試薬剤はBuddemeyer法には0.2N-NaOHで溶解後、pH7.0のphosphate buffered saline（PBS）で最終濃度が2.0、0.5μg/mlになるように調整したものを用いた。他方、ヌードマウス足蹠法は抗菌薬を所定の濃度（10、30、50、60、150mg/kg）になるよう0.001%-Tween 80含有PBS（pH 7.0）で調整後、冷凍庫（－30℃）中で保存し、使用に臨み室温で溶解したものを使用した。

4）抗らい菌活性の測定

①Buddemeyer法[14,15]：4-mlのガラスバイアル中にMiddlebrook-7H12培地、らい菌（$1x10^7$）、抗菌薬（最終濃度：2.0及び0.5μg/ml）を加えよく混和する。このガラスバイアルのキャップを緩く締め、32℃の炭酸ガスフラン器で4日間培養後、^{14}C-パルミチン酸（56mCi/mmol, DuPont NEN）を1μCi加えて混和後、NaOH-シンチレータで処理をしたろ紙片を入れた18-mlポリエチレンバイアルに入れキャップを強く締める。そして32℃の培養器中で7日間培養を継続し、産生した$^{14}CO_2$量を液体シンチレーションカウンターで測定し、NQの抗らい菌活性を比較検討した。

②ヌードマウス足蹠法：ヌードマウスの両後肢足蹠に1足蹠当たり10^7のらい菌を接種した。菌接種後3ヵ月から5ヵ月の3ヵ月間に亘り、ステンレスカテーテルで各抗菌薬を毎日週5日経口投与した。菌接種後8ヵ月から11ヵ月まで月1回、4ヶ月間、1群2匹4足蹠内のらい菌数を計測し、NQの最小抑制量を求めた。

結　果

1）表1に示したようにBuddemeyer法では、2.0μg/mlの濃度域でのNQの抗らい菌活性はSTFX ＞ WQ-3402 ≧ MFLX ＞ SPFX ＞ GFLX ＞ GRNX ≒ LVFX ＞ OFLXの順であった。

2）表2に示したようにヌードマウス足蹠法では、SPFXとMFLXは10mg/kgで最も強く、STFXとGFLXは30mg/kg、LVFXとGRNXは60mg/kg、OFLXは150mg/kgでヌードマウス足蹠内のらい菌の増殖を完全抑制したが、WQ-3402は、50mg/kg（製薬会社の合成部門の都合でこれ以上の濃度の実験ができなかった）で不完全抑制であった。

3）OFLX、LVFX、SPFX、GFLX、MFLX、GRNXの*in vitro*と*in vivo*抗らい菌活性間の相関係数は、-0.8593で強い負の相関が認められた。また*in vitro*と*in vivo*抗らい菌活性が一致しなかったSTFXとWQ-3402を加えた相関係数は、-0.7512で負の強い相関が認められた。

4）Buddemeyer 法とヌードマウス足蹠法の双方に強い抗らい菌活性を示した NQ は、キノロン母核 1 位にシクロプロピル基、3 位にカルボキシル基、4 位にオキシ基、5 位にアミノ基または水素基、6 位にフッ素基、7 位に 5 員環または 6 員環の塩基性環状アミン、8 位にフッ素基、塩素基またはメトキシ基を持つ MFLX、SPFX、STFX、GFLX であった。NQ 中で強い *in vitro*- 抗らい菌活性を示した WQ-3402 の *in vivo*- 抗らい菌活性は弱かった。

考　察

ハンセン病の化学療法では、治療期間の短縮と薬剤耐性に対応するため新規抗らい菌薬の開発が求められている。しかし、抗らい菌活性を示す抗菌薬は、多剤併用療法で用いられている DDS、B663、RFP とニューキノロン系抗菌薬（NQ）の SPFX、GFLX、MFLX など、マクロライド系の clarithromycin、テトラサイクリン系の minocycline に限られている。特にらい菌に対し殺菌作用を示し、多剤耐性菌に対し強い抗らい菌活性を示す NQ は、ハンセン病の治療薬として重要である。しかし、新世代 NQ であっても、強い抗らい菌活性を示すとは限らない。そこで NQ の構造式と抗らい菌活性の相関を検討した。今回実験に用いた NQs は、以下のような構造式的特徴を有する。

ofloxacin（OFLX、第一三共）[2] は、キノロン骨格にオキサンジン環し、7 位にメチルピペラジ

Table 1. *In vitro* activities of new quinolones against *M. leprae* measured by the method of Buddemeyer.

Drug	inhibition(%)[a] 2.0 μg/ml	0.5 μg/ml
OFLX	25.0±1.47	16.2±4.08
LVFX	31.1±0.87	19.8±1.64
SPFX	56.9±1.18	38.0±1.37
STFX	67.2±1.43	41.9±1.06
GFLX	47.7±1.12	23.6±1.31
WQ-3402	60.9±2.63	35.5±0.56
MFLX	60.2±0.74	41.6±1.54
GRNX	31.2±1.56	18.9±2.14

a: cpms of (control group) – (drug group) / control group x 100(%)

Table 2. Antibacterial activity of new quinolones against *M. leprae* inoculated into footpads of nude mice.

Drug	MID (mg/kg)
OFLX	150
LVFX	60
SPFX	10
STFX	30
GFLX	30
WQ-3402	50>
MFLX	10
GRNX	60

MID: minimum inhibitory dose

Nude mice were infected in the each of hind footpads, with *M. leprae* ($1x10^7$) of a strain Thai-53. New quinolones were given by the gavage 5 times per week,between 3 to 5 months postinfection at a daily dose of 10, 30, 50, 60 or 150 mg/kg. At 8,9,10 and 11 months after inoculation, mice were killed and the numbers of AFBs in the 4 hind footpads of 2 mice were counted accoding to the methods of Shepard.

ニル基を有するNQである。levofloxacin（LVFX、第一三共）[3]は、ラセミ体であるOFLXは、化学構造中に1個の不斉炭素を有し、2つの光学活性S-(－)体、R-(＋)が1：1よりなり、そのうち抗菌活性本体S-(－)体がLVFXである。sparfloxacin（SPFX、大日本住友製薬）[4]は、キノロン骨格の1位にシクロプロピル基、5位にジメチルピペラジニル基、7位にフッ素基を導入し、8位へのフッ素基導入は、抗らい菌活性を強めるが光毒性を強める課題もある[5]。sitafloxacin（STFX、第一三共）[6]は、キノロン骨格の1位にフルオロシクロプロピル基、8位に塩素基、7位にスピロヘプタン基、8位に塩素基を導入したNQである。gatifloxacin（GFLX、杏林製薬）[7,8]は、キノロン骨格の1位にシクロプロピル基、7位にメチルピペラジニン基、8位にメトキシ基を導入した8-methoxyquinoloneである。WQ-3402（湧永製薬）[9]は、キノロン骨格の1位に5-アミノ-2、4-ジフルオロフェニル基、7位にメチルアミノ基を有するNQである。moxifloxacin（MFLX、バイエル薬品）[10]は、キノロン骨格の1位にシクロプロピル基、7位にピロロピリジン基、8位にメトキシ基を導入した8-methoxyquinoloneである。garenoxacin（GRNX、富山化学工業）[11]は、従来NQの抗菌活性に必須とされたキノロン骨格の6位にフッ素基が無く、7位にインドール基、8位にジフルオロメトキシ基を導入し、既存ニューキノロン系薬と異なった構造式を有するNQである。

Buddemeyer法では、STFX、WQ-3402、MFLX、SPFX、GFLXの抗らい菌活性は強かったがLVFX、GRNX、OFLXの抗らい菌活性は弱かった（これらの実験は複数回行い、同様の結果が得られている）。またヌードマウス足蹠法ではSPFX、MFLX、STFX、GFLXの抗らい菌活性は強かったがLVFX、GRNX、WQ-3402、OFLXの抗らい菌活性は弱かった。Buddemeyer法とヌードマウス足蹠法の双方に強い抗らい菌活性を示したNQは、MFLX、SPFX、STFX、GFLXであった。

WQ-3402の*in vitro*活性は強いが*in vivo*活性が弱い理由として、血中半減期が2.7時間（犬、10mg/kg単回経口）と短く、蛋白結合率が高いなど体内動態が劣ることが考えられる[16]。また8位

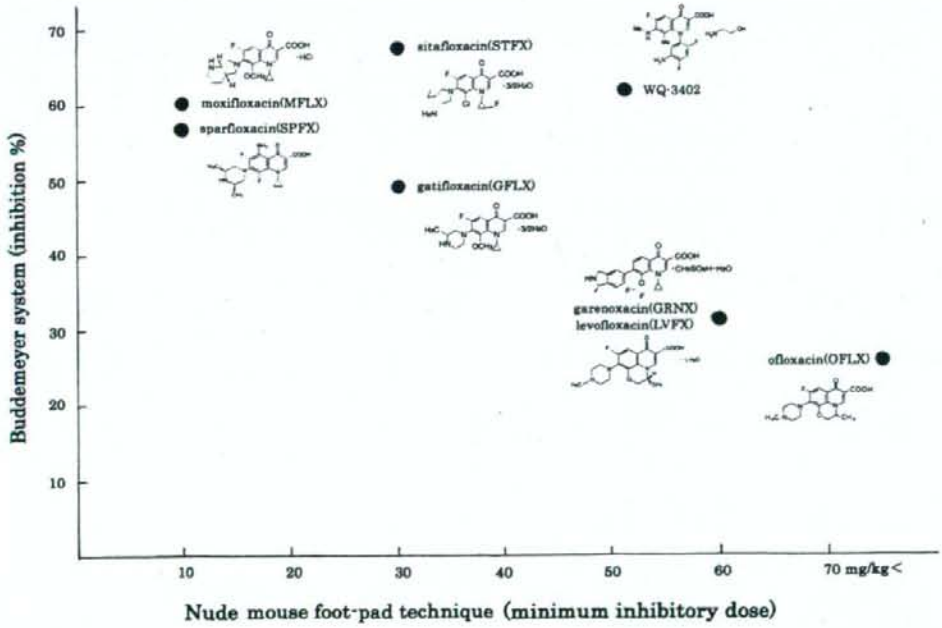

Fig. 1. *In vitro* and *in vivo* activities of new quinolones against *M. leprae*.

OCH_3、ClよりF基置換は、脂溶性を増し、組織移行性、腸肝循環、代謝安定性を高め、長い血中半減期を持つことで強い抗らい菌活性を示すと考えられる。

1位のシクロプロピル基は抗らい菌活性を増大、6位フッ素基導入は代謝安定性と抗らい菌活性を増大、8位のF、Clのハロゲン基またはメトキシ基導入は組織移行性と抗菌活性の増大を図るなどの薬理学的構造特性がある。さらに6位のハロゲン基は脂溶性、7位の塩基性環状アミンは親水性で双性イオン型構造であることから体内で適度な分配係数を示し、さらに7位の塩基性環状アミンは塩基性で3位のカルボキシル基は酸性で、これら双性イオン型構造は、菌体膜透過性、組織移行性、代謝安定性を高めることで強い抗らい菌活性を示すものと考えられる[17-20]。

以上の結果からキノロン母核の1位にシクロプロピル基、3位にカルボキシル基、4位にオキシ基、5位にアミノ基または水素基、6位にフッ素基、7位に5員環または6員環の塩基性環状アミン、8位にハロゲン基（フッ素基、塩素基）またはメトキシ基に置換したNQが、抗らい菌活性を最も強めることが示唆された。

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Structure and anti-*M.leprae* activity relationships of new quinolones

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Key words : anti-*M.leprae* activity, Buddemeyer system, new quinolones, nude mouse footpad method, structure-activity relationship

Due to the emergence of drug resistant *M.leprae*, there is a need to look for new drugs for the treatment of leprosy. We evaluated the effectiveness of new quinolones *in vitro* as well as *in vivo*.

The *in vitro* and *in vivo* results suggested that a cyclopropyl group at the 1-position, COOH at the 3-position, OH at the 4-position, NH_2 or OH-substitutions at the 5-position, F at the 6-position, 5- and 6-membered rings at the 7-position, halogen (F or Cl) or OCH_3 at the 8-position of the quinolone core structure, remarkably enhance ant-*M.leprae* activities of the drug.

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