doxycycline, and minocycline for both clinical isolates, the environmental isolate, and the *M. massiliense* reference strain were much lower than those for the *M. abscessus* and *M. bolletii* reference strains. Reinvestigation of the genotypic and drug susceptibility characteristics of the *M. chelonae-M. abscessus* group is needed. However, some differences in drug susceptibilities have been described that may allow clinicians to differentiate *M. massiliense* from other mycobacteria in the *M. chelonae-M. abscessus* group and to design specific therapies targeting the organism (1, 11, 16). Further study is needed to document the clinical features of, and treatment options for, cutaneous *M. massiliense* infection.

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PLoS NEGLECTED TROPICAL DISEASES

A Lipopeptide Facilitate Induction of *Mycobacterium leprae* Killing in Host Cells

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Abstract

Little is known of the direct microbicidal activity of T cells in leprosy, so a lipopeptide consisting of the N-terminal 13 amino acids lipopeptide (LipoK) of a 33-kD lipoprotein of *Mycobacterium leprae*, was synthesized. LipoK activated *M. leprae* infected human dendritic cells (DCs) to induce the production of IL-12. These activated DCs stimulated autologous $CD4^+$ or $CD8^+$ T cells towards type 1 immune response by inducing interferon-gamma secretion. T cell proliferation was also evident from the CFSE labeling of target $CD4^+$ or $CD8^+$ T cells. The direct microbicidal activity of T cells in the control of *M. leprae* multiplication is not well understood. The present study showed significant production of granulysin, granzyme B and perforin from these activated $CD4^+$ and $CD8^+$ T cells when stimulated with LipoK activated, *M. leprae* infected DCs. Assessment of the viability of *M. leprae* in DCs indicated LipoK mediated T cell-dependent killing of *M. leprae*. Remarkably, granulysin as well as granzyme B could directly kill *M. leprae in vitro*. Our results provide evidence that LipoK could facilitate *M. leprae* killing through the production of effector molecules granulysin and granzyme B in T cells.

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Introduction

The introduction of multidrug therapy in 1982 and the WHO campaign for the 'elimination of leprosy as a public health problem', have contributed greatly to the decrease in the prevalence rate over the past three decades. But leprosy still remains to be a public health problem in some countries, and the number of new cases detected during the last three years, remain steady [1]. The disease presents as a clinical spectrum that correlates with the level of the immune response to the pathogen [2]. Patients with lepromatous form of the disease have poor cellular immunity, resulting in extensive intracellular proliferation of *Mycobacterium leprae* bacilli in the skin and nerves. On the other hand, patients with the tuberculoid form of the disease are relatively resistant to the bacilli, so that few, if any, demonstrable bacilli are seen in the lesions [2,3]. For patients with abundant bacilli, whose lesions are characterized by type-2 cytokines, there is a need to up-regulate the T-cell mediated type 1 immune responses, by immunotherapeutic means to kill the bacilli.

We have previously identified a lipoprotein of *M. leprae*, a 33-kD lipoprotein (ML0603) [4]. Truncated protein, having the N-terminal 60 amino acids of 33-kD lipoprotein, had cytokine inducing ability in human monocytes, in contrast to the C-terminal 192 amino acids having no such ability [5]. In this study, we synthesized the lipopeptide (LipoK) having the N-terminal 13 amino acids of the 33-kD *M. leprae* lipoprotein linked to tri-palmitoylated portion of a lipid. Since GC mass spectrometry of mycobacterial lipoproteins provided evidence for the presence of three fatty acids (either palmitic, stearic or tuberculostearic acid), we assumed that tri-palmitoylated peptide would represent the natural lipoprotein of *M. leprae* [6,7]. Further, N-acyl transferase (Lnt) activity was identified in mycobacteria, which transfers the amide-linked acyl group to the N-terminal cysteine residue [6]. This presence of Lnt activity would indicate the presence of triacylated lipoproteins in mycobacteria, although the exact lipid structure of *M. leprae* lipoprotein is still to be determined. Previously, it was observed that hexameric peptides with tri-palmitoyl modification, corresponding to 19-kD and 33-kD lipoproteins of *M. leprae*, partially activates cells through TLR2-TLR1 heterodimers [8,9]. Since dendritic cells (DCs) are the most potent antigen presenting cells capable of bacilli uptake, antigen presentation and initiating acquired immune responses, DCs were used as target antigen presenting cells, in the present study [10,11]. As expected, it was found that LipoK, delivered signals through TLR2, and activated *M. leprae* infected DCs to produce abundant IL-12, although, LipoK does not produce IL-12, in non-infected DCs. Several mechanisms are known to be involved in the clearance of intracellular bacteria, including interferon gamma (IFN-γ) release, apoptosis induction of the host cells and anti-microbial activity of $CD8^+$ cytotoxic T lymphocytes (CTL) [12–15]. CTL mediated killing of mycobacteria, was demonstrated in tuberculosis by Thoma-Uszynski *et al.* They showed that $CD8^+$ CTL-mediated killing of *M. tuberculosis* was dependent on granule exocytosis [16].

In the present study, we analyzed whether *M. leprae* infected DCs, activated through LipoK could undergo functional changes and subsequently induce type 1 T cell activation to kill the bacilli. We observed that LipoK is a potent inducer of T cells equipped

Author Summary

We observed that LipoK (*Mycobacterium leprae* lipopeptide with 13 amino acids) is capable of inducing a good immune response in *M. leprae* infected human dendritic cells (DCs). These activated DCs had up-regulated expression of costimulatory molecule CD86 as well as CD83 (well known maturation marker) on their surface, and secreted IL-12, which is an important cytokine involved in the host defense against pathogens. Importantly, these mature DCs were capable of further driving type 1 responses by stimulating $CD4^+$ T cells and $CD8^+$ T cells for proliferation and interferon-gamma production. Further, both subsets of T cells were capable of producing cytotoxic granules: granulysin and granzyme B. *In vitro* experiments proved that these molecules are capable of killing *M. leprae* directly. It is the first report of the type, which proves that granulysin as well as granzyme B could partially kill *M. leprae*. LipoK would facilitate in inducing the immune responses in patients' harboring *M. leprae*.

with cytolytic function, which can largely contribute to the killing of *M. leprae* in host cells.

Materials and Methods

Ethics statement, cell culture and preparation of the bacteria

Peripheral blood was obtained from healthy Japanese individuals under informed consent. But no information of the donor (exposure to bacilli) was provided. In Japan, BCG vaccination is compulsory for children (0~4 years old). Monocyte-derived DCs were differentiated from monocytes using GM-CSF and IL-4 as described earlier [17,18]. Animal studies were carried out in strict accordance with the recommendations from Japan's Animal Protection Law. The protocol was approved by the Experimental Animal Committee, of the National Institute of Infectious Diseases, Tokyo (Permit Number: 210001). *M. leprae* (Thai-53 strain) is passaged in athymic *nu/nu* mice (Clea Co, Tokyo) [19]. At 8 to 9 months post-infection, the footpads were processed to recover *M. leprae* [20]. For all experiments, *M. leprae* was freshly prepared. The multiplicity of infection (MOI) was determined based on the assumption that DCs were equally susceptible to infection with *M. leprae* [21], and immature DCs were infected with *M. leprae* at MOI 50 in all experiments. Human cells without the bacilli was cultured at 37°C, but when infected with the bacilli, the cells were cultured at 35°C, which is the minimal temperature at which the cells can survive in *in-vitro* experiments. LipoK having the structure Palmitoyl-Cys((RS)-2,3-di(palmitoyloxy) -propyl)-Leu-Pro-Asp-Trp-Leu-Ser-Gly-Phe-Leu-Thr-Gly-Gly-OH, was synthesized by Bachem (Bubendorf, Switzerland). Using LAL assay (QCL-1000, Lonza), endotoxin was undetectable in original LipoK preparation (50 μg/ml). Therefore, any contaminating LPS in the synthesized product could be ruled out. Monoclonal Ab to TLR2 was kindly provided by Genentech, and mAb to mannose receptor and DC-SIGN were obtained from BD Biosciences. Parthenolide obtained from Santa-Cruz was used at a concentration of 2 and 5 μM. CD40L (Pepro Tech) was used at the concentration of 1 μg/ml, whenever needed.

Analysis of cell surface Ags on DCs and measurement of IL-12 production

Immature DCs were stimulated with *M. leprae* and/or LipoK for 48 hours. The expression of cell surface antigens on DCs, were analyzed using FACSCalibur flow cytometer (BD Biosciences). Dead cells were eliminated from the analysis by staining with 7-amino actinomycin D stain. For analysis of cell surface Ag, the following mAb were used: FITC-conjugated mAb against HLA-ABC (G46-2.6), HLA-DR (L243) and CD86 (FUN-1), purchased from PharMingen, and CD83 (HB15a, Immunotech). The ability of DCs to produce IL-12 on stimulation with either LipoK and/or *M. leprae*, was assessed. DCs were stimulated with the Ags on day 4 after the start of culture from monocytes. After 24 hours, OptEIA Human IL-12 (p70) ELISA Set (BD Biosciences) was used to determine the concentration of IL-12 p70 in the culture supernatant.

DC-T cell co-cultures

The ability of *M. leprae*-infected DCs to stimulate T cells was assessed using an autologous DC-T cell co-culture. $CD4^+$ T cells and $CD8^+$ T cells were purified using respective T cell enrichment Set (BD IMag) from freshly thawed PBMCs. The purity of $CD4^+$/$CD8^+$T cells was determined to be more than 95%. The purified responder cells (1×10^5 per well) were plated in 96-well round-bottom tissue culture plates, and mitomycin C-treated DCs which were pulsed with Ag, were added to give the indicated DC: $CD4^+$ or $CD8^+$ T cell ratio. Supernatants of DC-T cell co-cultures were collected on day 4, and IFN-γ production was measured by ELISA, using Opt EIA Human IFN-γ ELISA Set (BD Biosciences). In other experiments, Ag-pulsed DCs were treated with mAb to HLA-ABC (W6/32), HLA-DR (L243), CD86 (IT2.2) or normal mouse IgG. For obtaining naïve T cells, anti-CD45RO mAb (Dako) and anti-mouse IgG Ab Dynabeads M-450 (Invitrogen) were used to negatively select the cells. Since BCG is compulsory for children in Japan, it is likely that naïve T cells respond to *M. leprae* antigens, some of which are cross reactive to *M. bovis* BCG.

Measurement of T cell proliferation by CFSE labeling

DCs stimulated with Ags were co-cultured with the CFSE labeled total T cells. CFSE (Molecular Probes) was added at the concentration of 1 μM and incubated at 37°C for 10 min and stabilized according to the manufacturers' protocol. A total of 1×10^6 cells/well were seeded in a 24-well plate at a DC:T cell ratio of 1:6. After 8 days co-culture, cells were co-stained with PE conjugated anti-CD4 mAb and APC conjugated anti-CD8 mAb (BD Biosciences). CFSE signal of gated T cells were analysed.

Confocal microscopy

Imaging of cells was performed using laser scanning microscope LSM5-Exciter (Carl Zeiss). DCs grown on a 13-mm coverglass in a 24-well plate, were infected with *M. leprae* and/or stimulated with LipoK for 48 hours. T cell from the same donor was purified using the Dynal T cell isolation kit, and co-cultured with DCs for additional 3 days, after washing out extracellular bacilli. Cells were fixed in 2% paraformaldehyde, and the bacilli stained with 0.01% auramine O as described [22]. Anti-*M. leprae* membrane (minus LAM) polyclonal antibody was kindly provided by Dr. John S. Spencer through the NIH/NIAID Leprosy Research Support (N01 A1-25469). Fixed cells were blocked with normal human IgG (10 μg/ml), and stained with the above polyclonal antibody (1 μg/ml) for 30 min in PBS containing 0.1% saponin and 0.5% BSA, and the secondary antibody used was Alexa Fluor 633-conjugated goat anti-rabbit IgG (Molecular Probes), and images were recorded on fluorescent confocal microscope using a 63× oil objective, 488-nm and 633-nm lasers. Data was processed using the LSM software ZEN 2007. All bacilli observed were not surface attached as observed by section scanning (Z-stack Navigation).

Determination of intracellular levels of perforin, granzyme B and granulysin

After 7 days co-culture of purified T cells with DC pulsed with *M. leprae* and/or LipoK, intracellular detection of cytolytic effector molecules was performed. Briefly, GolgiStop (BD Biosciences) was added to the media for the last 12 hours of culture. Cells were first surface stained, fixed, permeabilized, and finally stained with FITC conjugated anti-perforin mAb or anti-granzyme B mAb or isotype control IgG2a (BD Biosciences). For the determination of intracellular levels of granulysin, the procedure was followed as for the intracellular stain of perforin, except that the surface stain used was FITC conjugated-CD4 and APC conjugated anti-CD8 mAb (BD Biosciences), and subsequently PE conjugated granulysin (eBioscience, GmbH, Germany) was used to determine the percentage of granulysin producing cells.

Determination of *M. leprae* viability in DCs

Since *M. leprae* cannot be cultured in vitro, we measured the viability of the bacilli, by the measurement of radioactive CO_2 production from oxidation of palmitic acid as described previously [23]. DCs were infected with *M. leprae* with or without LipoK, and co-cultured with T cells in some cases. Six days later, cells were harvested and washed 3 times in PBS, and centrifuged, so that *M. leprae* that might have escaped from the DCs into the media could be eliminated from our assay. Cell lysates were prepared as follows: 0.1 N NaOH solution was added to the cells for few minutes and then neutralized with the equal volumes of 0.1 N HCl solution. Subsequently, equal volume of 2 times concentrated Middlebrook 7H9 broth supplemented with ADC was added. ^{14}C labeled palmitic acid was added to the lysates of DCs and cultured at 33°C. Seven days later, the amount of $^{14}CO_2$ evolved and trapped on the filter paper was measured using a Packard 1500 TRI-CARB liquid scintillation analyzer. In a likewise manner, direct effect of *M. leprae* killing was observed by incubation of the bacilli with 3 μg/ml of granulysin (R&D systems) or granzyme B (Calbiochem) for a period of 3 days at 33°C, and then ^{14}C labeled palmitic acid was added to determine the viability as described above.

Statistical analysis

The unpaired student's t test was used to find the significance of the two sets of data. Differences were considered as statistically significant if $p<0.05$. All experiments were performed at least 3 times with different blood donors, unless otherwise stated, and the reproducibility of the experiment was evaluated. In some cases, ANOVA was used for probability calculation.

Results

LipoK activated human dendritic cells

We investigated the effect of LipoK stimulation on human monocyte derived DCs. All DCs were CD1a positive and CD14 negative [21]. When LipoK was used as a stimulant for immature DCs, maturation of DCs was observed as shown in Fig. 1. Up-regulation in the expression of CD83 (maturation marker of DCs) and CD86 (co-stimulatory molecule) was observed in LipoK stimulated DCs, the level of which, was similar to that of *M. leprae* infected DCs. *M. leprae* was used at the multiplicity of infection (MOI): 50 in all the experiments. The expression of the CD83 and CD86 molecules was more pronounced when LipoK was used to stimulate *M. leprae* infected DCs. The expression of HLA-ABC and HLA-DR molecules was not significantly different in LipoK stimulated *M. leprae* infected DCs from non-infected DCs, after 48 hours. Although, at earlier time points (18 hours after stimulation with antigen), a higher expression of HLA-ABC and

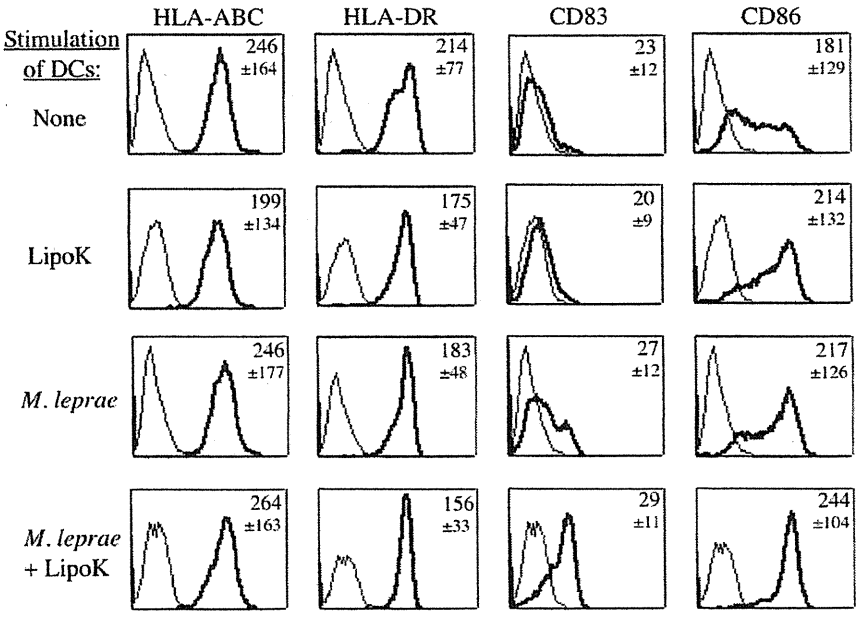

Figure 1. Expression of the surface markers on DCs after stimulation of *M. leprae* infected DCs with LipoK. The expression of cell surface markers on DCs, was analyzed using FACSCalibur. Dead cells were eliminated from the analysis by staining with 7-amino actinomycin D (7-AAD) stain. LipoK was used at a concentration of 0.3 μg/ml. The following mAb were used: FITC-conjugated mAb against HLA-ABC, HLA-DR, CD83 and CD86. Black light lines, isotype-matched control IgG. Black solid lines show the fluorescence intensity of the respective surface markers of DCs. Numbers indicate the mean fluorescence intensity with SD of the respective surface markers. Representative data of three separate experiments with different donors is shown.
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HLA-DR is observed in LipoK stimulated *M. leprae* infected DCs compared to non-stimulated.

Alternatively, when the IL-12 p70 secreted by DCs was measured, increasing dose of LipoK on *M. leprae* infected DCs produced the cytokine, with maximal cytokine production at LipoK concentration of 0.3 μg/ml (Fig. 2A). LipoK alone did not produce statistically significant amounts of IL-12 at the concentration of 0.3 μg/ml compared to the non-stimulated DCs. Another TLR-2 agonist, peptidoglycan could produce IL-12 (data not shown), probably due to the heterogeneous nature of the peptidoglycan which contains long peptide linkages. LipoK probably need other protein/peptide molecules to activate IL-12 production in DCs. Also, *M. leprae* infection alone did not produce IL-12 in DCs. When CD40 ligand (CD40L) was used to stimulate *M. leprae* infected DCs, IL-12 production was negligible.

As could be expected, TLR-2 antagonistic Ab completely blocked IL-12 production, whereas mannose receptor Ab did not, suggesting that IL-12 production from LipoK stimulated *M. leprae* infected DCs was TLR-2 dependent (Fig. 2B). When DCs were pre-treated with parthenolide, which is known to inhibit NF-kB activity [24], it was found that both 2 μM and 5 μM could significantly inhibit the production of IL-12 in a dose-dependent manner (Fig. 2C), indicating that NF-kB is involved in the IL-12 production from these LipoK stimulated DCs.

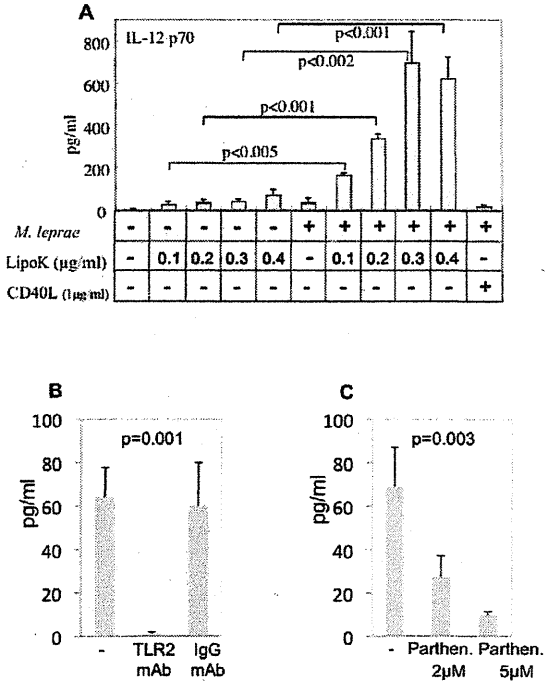

Figure 2. Production of IL-12 p70 from DCs. (**A**) Enhanced induction of IL-12 p70 from DCs by stimulation with LipoK and *M. leprae*. DCs were stimulated with the antigens on day 4 after the start of culture from monocytes. After 24 hours, IL-12p70 concentration in the culture supernatant was measured by the enzyme assay kit Opt EIA Human ELISA Set. The antigens used for the stimulation were: *M. leprae* and LipoK at different concentrations 0.1 μg/ml, 0.2 μg/ml, 0.3 μg/ml, and 0.4 μg/ml, CD40L was used at 1 μg/ml. (**B**) IL-12 p70 production from LipoK stimulated *M. leprae* infected DCs, is inhibited by antagonistic antibodies to TLR-2, and not by control normal IgG. (**C**) Effect of 2 μM and 5 μM of parthenolide (parthen.) on the IL-12 production was observed. Representative data of three separate experiments with different donors is shown. The probability by ANOVA was calculated to be 0.001 for (B) and 0.003 for (C).
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LipoK pulsed human DCs activated human T cells *ex vivo*

To investigate the effect of LipoK on T cell responses, purified $CD4^+$ and $CD8^+$ T cells from autologous donors were cultured with activated DCs. IFN-γ release was measured as correlates of T cell activation. When the IFN-γ levels were compared, DCs activated with *M. leprae* and LipoK produced significantly higher dose of IFN-γ from $CD4^+$ T cells, when compared to that produced by DCs stimulated with *M. leprae* or LipoK alone, or *M. leprae* in presence of CD40L (Fig. 3A), at both high (T:DC = 20:1) and low (T:DC = 40:1) dose of DCs. Note that *M. leprae*-infection or LipoK-stimulation alone was not efficient in stimulating T cells. Similarly, secretion of IFN-γ was also observed from $CD8^+$ T cells but at lower level compared to that from $CD4^+$ T cells. Again there was significantly high production of IFN-γ from $CD8^+$ T cells co-cultured with LipoK stimulated *M. leprae*-infected DCs compared to that from CD40L stimulated *M. leprae*-infected DCs (Fig. 3A). Although the IL-12 p70 production differed in LipoK stimulated *M. leprae*-infected DCs and CD40L stimulated DCs, no IL-12 production was observed from these mitomycin treated DCs which were co-cultured with T cells. In addition, as shown in Fig. 3B, although normal murine IgG did not affect the T cell stimulating activity of both $CD4^+$ and $CD8^+$ T cells, mAbs to HLA-ABC and HLA-DR, inhibited $CD8^+$ T cells and $CD4^+$ T cell activation of LipoK-stimulated *M. leprae*-infected DCs' respectively. The results indicated that the activation of these T cells were MHC Class II- and Class I-dependent in $CD4^+$ T cell and $CD8^+$ T cells respectively. The inhibition was comparable to that of inhibition of IFN-γ production by mAb to co-stimulatory molecule CD86.

Proliferation of these LipoK activated $CD4^+$ and $CD8^+$ T cells, was confirmed by the CFSE labeling of T cells. The labeling experiment was preferable because it could measure proliferation of individual T cell subsets even in the presence of the other subsets. *M. leprae* stimulation of DCs resulted in proliferation of 39.7% of total $CD4^+$ T cells, but stimulation with both LipoK and *M. leprae* resulted in proliferation of 67.5% of total $CD4^+$ T cells. LipoK stimulation alone did not induce any significant proliferation of $CD4^+$ T cells (Fig. 3C). The profiles of flow cytometric analyses showed that 25.3% of $CD8^+$ T cells proliferated by stimulation with *M. leprae* alone, but higher number of cells proliferated (38.9%) in presence of LipoK stimulus.

Subsequently, we examined the response of naïve T cells to LipoK activated DCs. When naïve $CD4^+$ T cells were cultured with DCs activated with *M. leprae* and LipoK, significantly higher dose of IFN-γ was produced in comparison to those cultured with DCs stimulated with *M. leprae* alone or LipoK alone. Production of IFN-γ was low from those activated with *M. leprae* and CD40L (Fig. 3D). It was observed that the IFN-γ production from naïve $CD8^+$ T cells, co-cultured with DCs stimulated with *M. leprae* and LipoK was meager.

When *M. bovis BCG* was used for infecting DCs, the MOI of the bacilli had to be lowered to almost 1~10, because higher MOI (50) would kill the DCs in *in-vitro* culture. BCG when infected at MOI:1 produced 156 pg/ml of IFN-γ from CD8 T cells, but when LipoK was used to stimulate BCG infected DCs, the amount of IFN-γ increased to 380 pg/ml, indicating that LipoK could lead to further T cell activation of BCG infected DCs. It is also likely that LipoK stimulation could increase the production of perforin and granulysin in *M. tuberculosis* infected host cells.

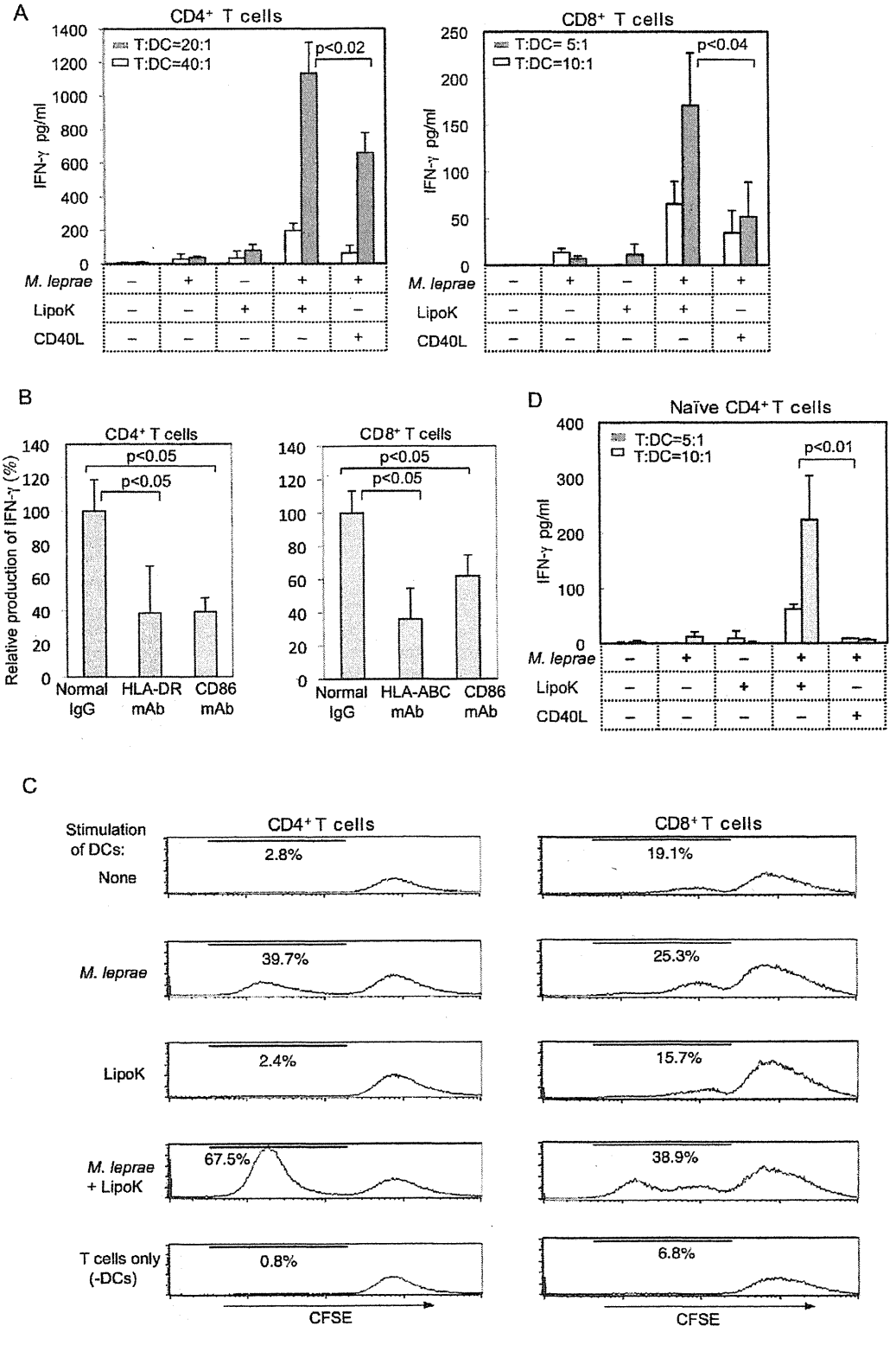

Figure 3. T cell activity as determined IFN-γ production and T cell proliferation. (**A**) The effect of LipoK on *M. leprae*-infected DCs to stimulate T cells was assessed using an autologous DC-CD4$^+$ or DC-CD8$^+$ T cell co-culture. IFN-γ production in the supernatant was measured by ELISA, after 4 days co-culture. (**B**) Effect of normal murine IgG or mAb to HLA-ABC/HLA-DR or CD86 on IFN-γ production from T cells co-cultured with *M. leprae* infected DCs simulated with LipoK. The production of IFN-γ from Ab non-treated T cells, cultured with LipoK and *M. leprae* stimulated DCs, is considered 100% and the actual value of IFN-γ produced from CD4$^+$ T cells is 250 pg/ml and that from CD8$^+$ T cells is 47 pg/ml at T cell:DC ratio of 10:1. (**C**) Proliferation of CD4$^+$ and CD8$^+$ T cells as assessed by CFSE labeling of T cells. DCs were mixed with autologous CFSE labeled T cells at a T cell:DC ratio of 10:1. Proliferating T cells were analysed by FACSCalibur on day 7 after co-culture. The percentage of proliferated cells is indicated. The

lowest histogram shows unstimulated T cells. (**D**) IFN-γ production from DC-naïve $CD4^+$ T cell co-culture. IFN-γ production was measured after 4 days co-culture with stimulated DCs. Representative data of four separate experiments with different donors is shown. Assays were performed in triplicate and the results are expressed as the mean +SD.
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Up-regulation of perforin, granzyme B and granulysin production in $CD4^+$ and $CD8^+$ T cells

To determine whether cytotoxic effect could be induced in highly activated T cells, we analysed the intracellular production of perforin and granzyme B in DC co-culture system with unseparated T cells. As seen in Fig. 4A, 15.8% of activated $CD8^{high}$ T cells produced perforin and 24.9% produced granzyme B when stimulated with DCs activated with *M. leprae* and LipoK, in comparison to those co-cultured with DCs activated with *M. leprae*, showing 1.4% of perforin and 1.8% of granzyme B-producing T cells. Thus, prominent enhancement of both perforin and granzyme B producing $CD8^+$ T cells was observed. Recently, since $CD4^+$ T cells are also known to possess direct cytotoxic potential [25], we measured the percentage of $CD4^+$ T cells producing perforin and granzyme B. When LipoK and *M. leprae* stimulated DCs were co-cultured with T cells, 12.7% of $CD4^{high}$ T cells produced perforin and 14.6% of those cells produced granzyme B, whereas in presence of *M. leprae* stimulated DCs, 6.6% produced perforin and 8.3% produced granzyme B (Fig. 4B). These data indicated that in addition to $CD8^+$ T cells, $CD4^+$ T cells also had the capacity to produce significant amounts of perforin and granzyme B. Nevertheless, the percentage of $CD8^+$ T cells producing these cytolytic proteins was 1.2~1.7 fold higher than $CD4^+$ T cell. Then, we examined, whether $CD8^+$ T cells alone without the direct contact with $CD4^+$ could have the same capacity. When $CD4^+$ T cells were allowed to culture in inserts, so that there was no direct contact between $CD8^+$ and $CD4^+$ T cells, there was decreased production of both perforin (7.3% v/s 15.8%) and granzyme B (9.5% v/s 24.9%) producing $CD8^+$ T cells (Fig. 4A). So, a direct contact of $CD4^+$ and $CD8^+$ T cells was necessary for sufficient production of cytolytic proteins. When we examined whether exogenous IL-2 could substitute the action of $CD4^+$ T cells, we found that addition of 50 U/ml of IL-2 (excess amount) to $CD8^+$ T cells, could produce both perforin and granzyme B equivalent to that of $CD8^+$ T cells co-cultured with LipoK stimulated, *M. leprae* infected DCs in the presence of $CD4^+$ T cells. However such high levels of IL-2 cannot be produced from host cells, in our experimental setting.

The intracellular level of another cytolytic protein, granulysin, was then examined. Enhancement of granulysin producing $CD8^+$ T cells was observed when co-cultured with DCs activated with *M. leprae* and LipoK. As seen in Fig. 4C, 18.9% of activated $CD8^{high}$ T cells and 28.4% of activated $CD4^{high}$ T cells produced granulysin when co-cultured with DCs activated with *M. leprae* and LipoK, in comparison to those co-cultured with DCs activated with *M. leprae*, (1.7% of $CD8^{high}$ T cells and 0.6% of $CD4^{high}$ T cells).

Mycobacterium leprae components were observed at the periphery of the infected DCs stimulated with LipoK, and co-cultured with T cells

To examine the fate of *M. leprae* in activated DCs, the cells were stained with anti-*M. leprae* membrane polyclonal antibody. Confocal microscopy revealed rod shaped *M. leprae* as observed by auramine-O stain, and membrane components seem to be rather localized in the region where *M. leprae* are present (Fig. 5). Strickingly, those DCs stimulated with LipoK for 48 hours and co-cultured with T cells for additional 3 days showed membrane staining at the periphery of the DCs (Fig. 5 arrowheads shown), probably due to processing of the bacilli in activated DCs.

Killing of *M. leprae* in DCs, by the LipoK stimulation

We determined the viability of *M. leprae* in DCs after stimulation with LipoK in the presence of autologous $CD4^+$ and $CD8^+$ T cells. Since *M. leprae* is uncultivable *in vitro*, the viability of *M. leprae* in DCs, after co-culture with the T cells for a week, was determined by the radiorespirometric assay. The amount of radioactive CO_2 evolved which reflects the rate of ^{14}C-palmitic acid oxidized by *M. leprae*, was measured by the scintillation counter. No significant reduction in $^{14}CO_2$ production was observed, from DCs, not co-cultured with T cells, even in the presence of LipoK stimulation (Fig. 6A). But, when the bacilli were recovered from DCs stimulated with LipoK and co-cultured with T cells, $^{14}CO_2$ production were significantly lower ($p<0.001$) than those recovered from DCs not stimulated with LipoK or T cells. The result indicates that approximately 50% reduction in the viability of *M. leprae* was observed in LipoK activated DCs and co-cultured with T cells compared to those obtained from DCs not stimulated with LipoK (Fig. 6B), indicating that T cells were essential and LipoK stimulation to DCs, was necessary to kill *M. leprae* in DCs. To further determine whether the cytolytic granules namely, granulysin and granzyme B could directly kill *M. leprae*, the bacilli was incubated with human granulysin or granzyme B for a period of 3 days at 33°C. Statistically significant reduction of $^{14}CO_2$ was observed when the bacilli were incubated with granulysin as well as granzyme B (Fig. 6C).

Discussion

In the present study we investigated the role of *M. leprae*-derived synthetic lipopeptide (LipoK), which consists of N-terminal 13 amino acids of the 33-kD *M. leprae* lipoprotein (Accession no. ML0603) linked to Palmitoyl-Cys((RS)-2,3-di(palmitoyloxy)-propyl group in the induction of intracellular killing of *M. leprae* through immuno-activation. Previously, we observed that the 33-kD lipoprotein and the truncated form of the protein induced the production of IL-12 in human peripheral blood monocytes [4,5]. Although human DCs are potent inducers of acquired immune responses, when DCs were exposed to *M. leprae*, they are inefficient in activating T cells [21,26]. It is generally recognized that, stimulation of T cells by intracellular pathogens, such as mycobacteria, is achieved by the coordinated processing of the antigens in the phago-lysosome of APCs and the expression of the antigenic determinants on APCs. Furthermore, CD40-CD40L interaction on immature DCs, are known to contribute to cell mediated responses in leprosy [27,28]. In fact, when *M. leprae* infected DCs were stimulated with CD40L, up-regulation of CD83 and CD86 molecules was observed (not shown). However, we found that CD40L failed to induce the production of IL-12 p70 in *M. leprae* infected DCs. In contrast to CD40L stimulation, LipoK stimulation on *M. leprae* infected DCs induced significant production of IL-12. Further, the expression of CD40 on DCs was not enhanced by stimulating *M. leprae* infected DCs with LipoK. It was evident that IL-12 inducing ability of these matured DCs was mediated by TLR2, and not by other receptors such as mannose receptor or DC-SIGN, as observed in DCs exposed to *M. tuberculosis* or *M. bovis* BCG [29,30,31]. The TLR2 antagonistic

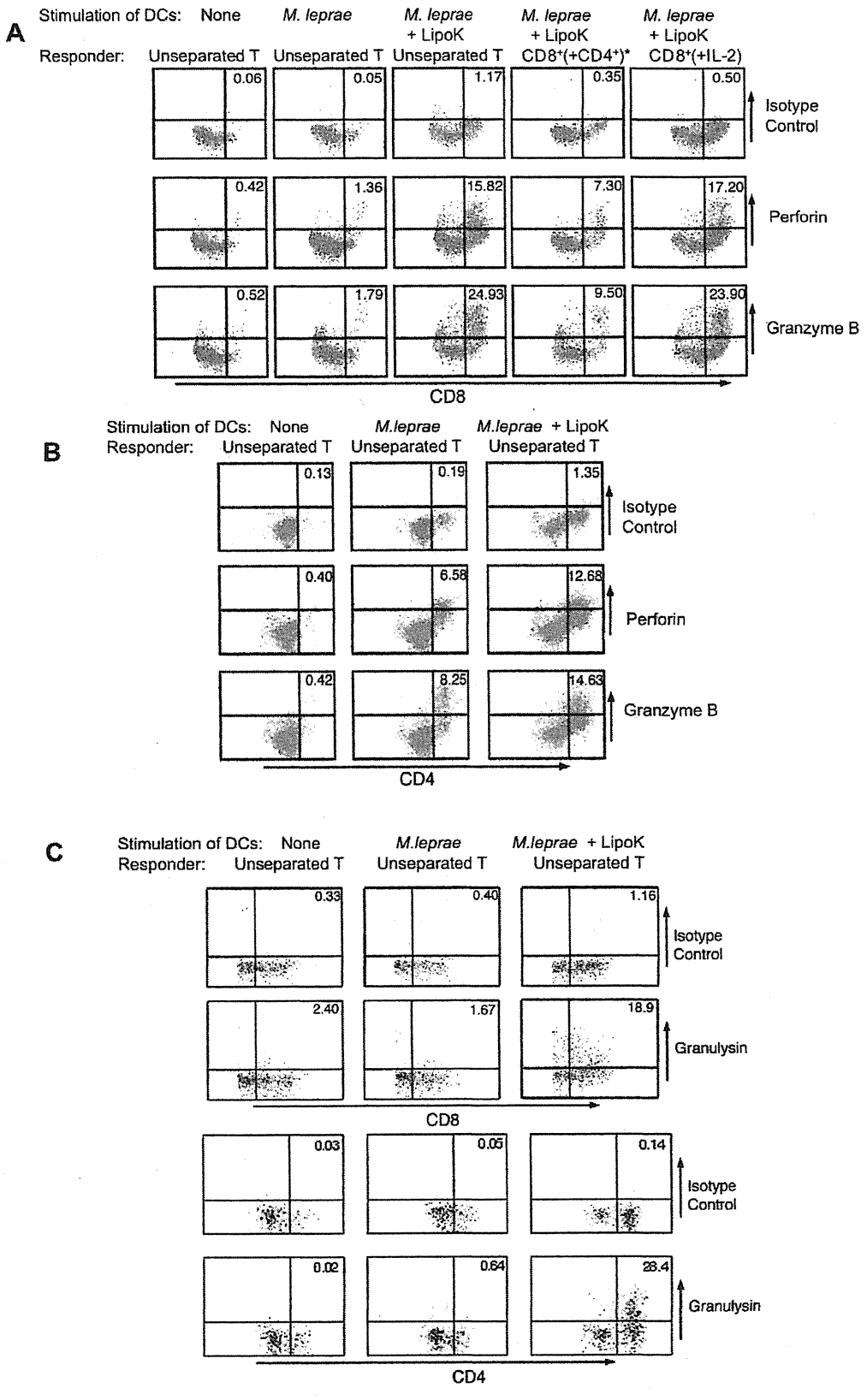

Figure 4. Production of perforin, granzyme B and granulysin in CD8$^+$ T cells as well as CD4$^+$ T cells. (**A**) Enhanced production of perforin and granzyme B from CD8$^+$ T cells cultured with LipoK stimulated *M. leprae* infected DCs. Intracellular staining of perforin and granzyme B was performed as follows: Cells were first stained with PE conjugated anti-CD4 or APC conjugated anti-CD8 mAb. Then, the cells were fixed in 2%

formaldehyde, permeabilized in 0.1% saponin, and stained with FITC conjugated anti-perforin mAb or anti-granzyme B mAb or isotype control IgG2a. Figure shows the dot plot of the gated $CD8^+$ T cells. The right hand quadrant shows $CD8^{high}$ T cells (activated $CD8^+$ T cells) and the number indicates the percentage of perforin or granzyme B positive T cells among gated $CD8^{high}$ T cells. *To determine whether direct interaction between $CD4^+$ and $CD8^+$ T cells for perforin and granzyme B production from $CD8^+$ T cells, is needed, $CD4^+$ T cells were cultured in inserts in a 24-well plate, and were not allowed to interact directly with $CD8^+$ T cells. As a control experiment, exogenous IL-2 (in the left hand dot plot) at a concentration of 50 U/ml was added to $CD8^+$ T cells. (**B**) Enhanced expression of perforin and granzyme B from $CD4^+$ T cells. The right hand quadrant shows $CD4^{high}$ T cells, and the number indicates percentage of $CD4^{high}$ T cells producing perforin and granzyme B. (**C**) Enhanced expression of granulysin from $CD8^+$ and $CD4^+$ T cells, co-cultured with LipoK and *M. leprae* stimulated DCs. The protocol was followed as per the staining of perforin, except that the surface stain used was FITC conjugated-CD4 and APC conjugated anti-CD8 mAb, and subsequently PE conjugated granulysin was used. Figure shows the dot plot of the gated $CD8^+$ and $CD4^+$ T cells. The right hand quadrant shows $CD8^{high}$ or $CD4^{high}$ T cells (activated T cells) and the number indicates the percentage of granulysin positive T cells among gated $CD8^{high}$ and $CD4^{high}$ T cells. Representative data of three separate experiments with different donors is shown.

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antibody could almost totally inhibit the IL-12 production from DCs, as well as the T cell activating function of DCs (not shown), probably through blocking of the classical NF-kB pathway. Indeed, parthenolide, one of the major sesquiterpene lactones, known to inhibit NF-kB activity [24], inhibited the IL-12 production from DCs stimulated with *M. leprae* and LipoK. Also, IL-12 was efficiently produced when *M. leprae* was viable and not dead. Thus, although the exact mechanisms remain to be elucidated, some cell surface molecules and secreted components of *M. leprae* are responsible for the production of IL-12, which further modulates type 1 T cell responses [32,33].

A number of mechanisms are known to be involved in the clearance of intracellular bacteria, such as IFN-γ release, apoptosis induction of the macrophages and anti-microbial activity of CTL [12,15]. Production of IFN-γ could boost the ability to kill pathogens in host cells. In fact, it was found that LipoK activated *M. leprae* infected DCs, highly stimulated both memory $CD4^+$ and $CD8^+$ T cells, as well as naïve $CD4^+$ to produce IFN-γ, and further assisted in the proliferation of both T cell subsets (Fig. 3).

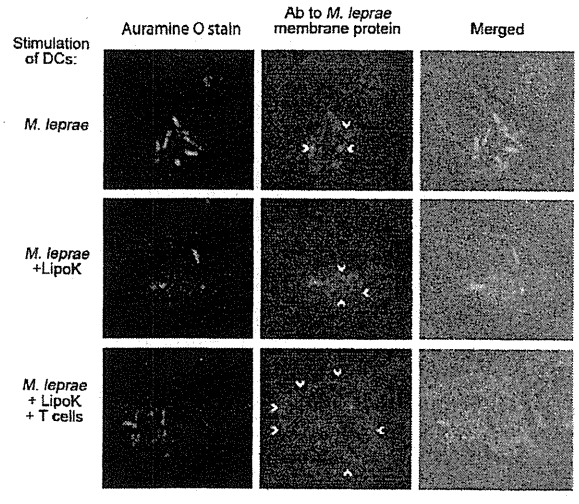

Figure 5. Localization of the membrane components of *M. leprae* at the periphery of the DCs. DCs were infected with either *M. leprae* alone or further stimulated with LipoK for 2 days and in some cases co-cultured with T cells. After 3 days co-culture with T cells, cover glass with attached DCs were fixed and observed under confocal microscopy-LSM5 Exciter. *M. leprae* was stained with Auramine O (shown in green) and *M. leprae* membrane components were stained with polyclonal rabbit antibody raised against the membrane fraction of *M. leprae* (depicted in red fluorescence). Alexa Fluor 633 conjugated anti-rabbit antibody (Molecular Probes) was used as the secondary antibody. Arrowheads indicate the positively stained region. Experiments were performed twice with different donors.

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Inhibition of MHC class I and class II molecules on DCs, indicated that the activation of these T cells were MHC class II- and class I-dependent in $CD4^+$ T cell and $CD8^+$ T cells respectively. Further, proteolytic processing of *M. leprae* antigens was probably enhanced by LipoK treatment of DCs, since incubation with anti-*M. leprae* membrane Ab showed positive staining at the periphery of DCs, when co-cultured with T cells (Fig. 5). In addition, preliminary results showed that expression of MHC class I and II molecules on LipoK activated DCs, were elevated in those co-cultured with T cells. Thus, LipoK could probably assist in the processing and presentation of *M. leprae* antigens, and thereby, highly activate T cells.

The other important parameter, for the clearance of mycobacteria from the host cell, is their potential to activate antimicrobial effector mechanisms in human T cells. DCs have been shown to be involved in CTL induction following uptake of antigenic particles [25,34,35,36]. $CD8^+$ T cells co-cultured with LipoK

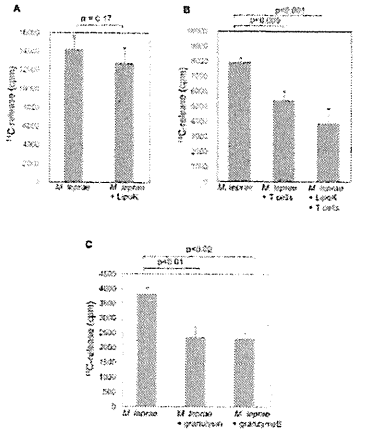

Figure 6. Reduction in the viability of *M. leprae* in DCs after co-culture with T cells and LipoK stimulation. (**A**) DCs were infected with *M. leprae* and stimulated with LipoK, 2 days later, cells were collected and the viability of *M. leprae* in DCs was measured by the radiorespirometric assay (metabolic CO_2 release) as described in Materials and Methods. In brief, ^{14}C labeled palmitic acid was added to the lysates of DCs and cultured at 33°C. After 7 days of culture, the amount of $^{14}CO_2$ evolved was measured using a Packard 1500 TRI-CARB liquid scintillation analyzer. (**B**) DCs were infected with *M. leprae* as in **A**, and co-cultured with T cells. Six days after the co-culture, DCs were lysed, and the viability of *M. leprae* was determined by the radiorespirometric assay. (**C**) *M. leprae* at a concentration of 1×10^7/well/200 µl in Middlebrook 7H9 media was incubated with granulysin or granzyme B for a period of 3 days at 33°C, and the viability determined as described in **A**. Unpaired Student's t test was used to find the statistical significance of the two sets of data. Representative data of three separate experiments is shown.

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stimulated *M. leprae*-infected DCs, through $CD4^+$ T cells' help produced increased amount of cytolytic effector molecules: perforin and granzyme B. Adequate production of these cytolytic proteins from $CD8^+$ T cells required direct contact with $CD4^+$ T cells. Recently, there are studies that certain types of $CD4^+$ T cells possess direct cytotoxic potential [25,37,38]. We observed a portion of $CD4^{high}$ T cells (activated T cells), have the capacity to produce cytotoxic granules. Bastian et al. demonstrated that native *M. tuberculosis* heterogenous lipopeptides are potent immunogens for primary human T cells, and those T cells were $CD4^+$ and MHC class II restricted, challenging the current concepts that cytotoxic T cells were restricted to $CD8^+$ T cell subset [25]. Another lytic molecule, present in cytotoxic granules of T cells, is granulysin, which is reported to have direct anti-bacterial activity. Reports have shown the ability of T cells to secrete granulysin at the site of *M. leprae* infection, which provides evidence that anti-microbial activity of granule containing T cells is a mechanism of host defense in leprosy [39,40]. We observed that LipoK stimulated, *M. leprae* infected DCs, highly enhanced the production of granulysin from $CD8^+$ T cells. Unexpectedly, we observed that the percentage of $CD4^+$ T cells producing granulysin was higher than $CD8^+$ T cells. But, this fact was in lines with the earlier data, which showed co-localization of granulysin and $CD4^+$ T cells in tuberculoid leprosy lesions [39,40]. Thus, granulysin release by LipoK-mediated activation process, may lead to a direct anti-microbial effector pathway of host defense. These data demonstrated that both $CD4^+$ T cells and $CD8^+$ T cells, contribute to the induction of intracellular killing of *M. leprae*. These speculations were further supported by the fact that 50% of the phagocytosed bacilli were killed when infected DCs stimulated with LipoK, were co-cultured with T cells. This is the first observation of killing of *M. leprae* in an *ex vivo* system using human DCs and T cells. To further provide evidence of the effector mechanism at work during *M. leprae* killing by CTL, the direct effect of granulysin on *M. leprae* killing *in vitro* was analyzed. Results indicated that about 40% of *M. leprae* was killed by granulysin. Granulysin could probably lyse *M. leprae* by binding to the lipidic cell wall, through the same mechanism by which *M. tuberculosis* is destroyed by granulysin. Since, perforin is an essential molecule in the killing of intracellular *M. tuberculosis* [16], similar operation may be involved in intracellular *M. leprae* killing since perforin was effectively produced by T cells in our CTL culture system. On the other hand, direct killing of mycobacteria by granzymes is not known. But the viability of *M. leprae* was significantly lowered by granzyme B. Since granzyme B is one of the serine proteases that can target cytosolic and nuclear substrates to induce host cell death through mitochondrial perturbation, it may be involved in destroying the cell wall architecture of *M. leprae* by still unknown mechanism [41,42]. The contribution of the cytotoxic granules to killing of bacteria remains to be of interest for further investigation. Together, the results indicate that LipoK could contribute to protective host response against leprosy and eventually kill the bacteria, through the production of perforin, granulysin and granzyme B in T cells.

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Author Contributions

Conceived and designed the experiments: YM TT YF MM. Performed the experiments: YM TT YF. Analyzed the data: YM TT TM MK MM. Contributed reagents/materials/analysis tools: YM TM MK. Wrote the paper: YM TT MM.

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WHO Global Leprosy Programmeによる薬剤耐性拠点監視事業における我々の役割

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キーワード：拠点監視事業、国際協力、ハンセン病、薬剤耐性

ハンセン病の化学療法と薬剤耐性

今日の世界のハンセン病対策はWHOにより推進され1981年に始まった多剤併用療法（MDT）を基本としている[1]。MDTの治療効果は高く、近年の世界のハンセン病有病率は顕著な減少を示している。しかしながら、化学療法とそれに使用される抗生物質に対する耐性の出現は、対象となる感染症の如何を問わず常に表裏一体の現象である。事実、1940年代にProminがハンセン病の治療に導入され、更に1949年にDapsoneが使用され始めてほどなく、再発症例あるいは難治症例でのDapsone耐性例が報告された[2,3]。その後Dapsone一次耐性の報告[4]、Rifampicinに対する耐性[5]、多剤同時耐性の報告がされた[5-8]。WHOによるMDT後の再発例が少なからず報告されているが[9]、それらにおける薬剤耐性、とりわけ強い殺菌作用を有し、MDTの中核をなすRifampicinに対する耐性は、今後のハンセン病の治療を危うくすることが懸念されている。東南アジア3カ国での調査ではフィリピンのセブの症例からはRifampicin耐性は見出されなかったが、インドネシア、ミャンマーではそれぞれ3.3%および1.3%の一次耐性がみられ、更に再発例では20%および8.3%のRifampicin耐性率が示され[10]、rifampicin耐性菌の存在が示された。

WHOによる薬剤耐性拠点監視事業の背景

このような状況下、前Global Leprosy Programme（GLP）のTeam leaderであったDr. Vijaykumar Pannikarは薬剤耐性について多数の検体を調査することにより包括的データに基づく耐性菌の動向をモニターし、その伝播状況の把握に基づいて、MDTを主たる手段とするハンセン病対策に耐性菌が及ぼす影響を検討することの必要性を認識していた。彼は報告がないことは耐性菌が存在しないことではなく、ただ検査が行われていないが故のことと考えた。しかしながら、らい菌の薬剤感受性検査に用いられるマウスfootpad法では、技術的な煩雑さからそのような要求を満たすことはできないために、目的とするデータの集積はほとんどなかった。その一方、彼は当時すでに1990年代より日本で行われていたDapsone, Rifampicin, Quinolone耐性を*folP1*, *rpoB*, *gyrA*遺伝子中のdrug resistance determining region（DRDR）における変異の検索により判定する手法[11,12]についての情報を持っていなかった。

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事業開始までの経緯とその後の会議

そのような折に、2006年5月にスコットランドのアバディーンで開催されたTechnical Adviser Group（TAG）の会議において畑野邑久光明園園長が、Dr. Pannikarから多数の例について検査可能な方法に基づいて、薬剤耐性菌の伝播を把握しうる簡易耐性菌検出技術について諮問された。これに対して、畑野園長は当時、筆者らが既に行っていた日本における難治例をはじめとするハンセン病治療薬に対する遺伝子変異に基づく薬剤耐性検査について具申した。その情報を受け、2006年11月にインドのアグラにおいて11ヵ国より16名が参加し、Informal Consultation on Rifampicin Resistance in Leprosy会議が行われた。この迅速な対応からして、GLPあるいはDr. PannikarがRifampicin耐性の問題をいかに重要視していたかがうかがわれる。松岡はDRDRにおける遺伝子変異とマウスfootpad法による耐性の関係、検体の保存・輸送方法、PCR Direct Sequencing方法とその適応例について報告し、薬剤耐性を遺伝子解析により検討することの正当性を示した。また、PCR Direct Sequencingの国内および東南アジア3カ国での臨床サンプルへの適用実績とそれらにおける耐性菌の伝播状況について報告した[10]。

上記会議において、下記事項が合意され、監視事業の開始が決定された。

1、調査対象国の事業実施可能な調査地点において継続的観察を行い、耐性菌の動向を把握する。

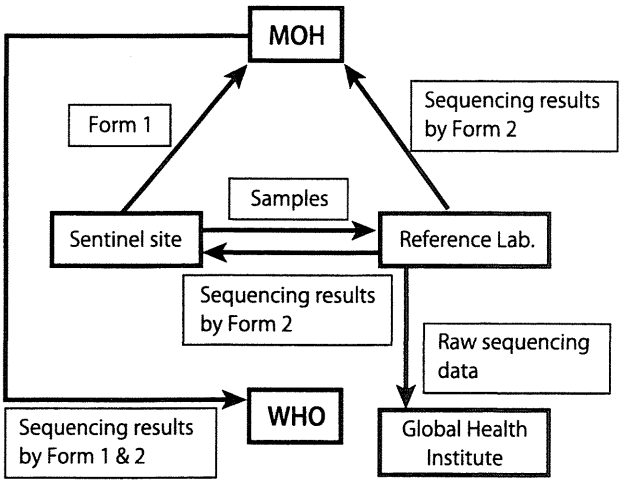

図1　WHOによる拠点監視事業体制の概要

2、WHOのMDT完了後、再発した症例について、Rifampicin、Dapsone、Quinolone耐性の有無を調査する。可能であれば新患例についても実施する。

3、*rpoB, folP1, gyrA*遺伝子のDRDRにおける遺伝子変異をPCR direct sequencingによって検索し、耐性の判定を行う。マウスfootpad法による検査は行わない。それらの方法についてはガイドラインに詳しく述べられている[13]。組織体制を図1に示した。それぞれの監視拠点で採取された検体は検査を担当するReference laboratoryに送付され遺伝子変異の検索が行われる。得られたデータは監視拠点に送られ、治療薬の選択に反映させるとともに、関係機関に送付される。

その後、2008年には第1回のワークショップがハノイで開催され、WHOおよび12ヵ国より40名が参加し、ついで2009年にWHOおよび20ヵ国、59名の参加の下に第2回の会議がパリにおいて開催された。さらに2010年11月9日及び10日の両日、国立感染症研究所において第3回の会議が開催され、筆者がその運営を担当した[14]。WHOおよび18ヵ国、42名の参加であった。それぞれの会議では各国のハンセン病対策の現状、再発症例の発生状況、それらの耐性検査の結果について報告され、合せて薬剤耐性に関する最新のトピックについての報告がされている。2011年現在の監視拠点はインド（3ヶ所)、ミャンマー（2ヶ所)、ベトナム（2ヶ所)、フィリピン（1ヶ所)、インドネシア（2ヶ所)、中国（1ヶ所)、コロンビア（1ヶ所)、ブラジル（5ヶ所)、マダガスカル（1ヶ所)、イエメン（1ヶ所)、エチオピア（2ヶ所)、マリ（1ヶ所）の12カ国に設定されている。収集された検体はインド、中国、インドネシア、ブラジルにおいてはそれぞれの国内の研究室において検査され、その他の国の検体については1）National Reference Center on Mycobacteria, Faculte de Madicine Pitie-Slpetriere, Paris, France 2）Department of Microbiology Yonsei University College of Medicine, Seoul, South Korea 3）Colorado State University, Fort Collins, Colorado, USA 4）Department of Microbiology, Global Health Institute, Ecole Polytechnique Federal de Lausanne, Lausanne, Switzeland 5）ハンセン病研究センターのいずれかが検査を行い、当センターの甲斐室長

がミャンマー、ベトナムからの検体の検査を担当している。

松岡はこれらreference centerの検査におけるQuality controlを担当している。2009年には8ヶ所のreference centerに対して検査におけるquality controlを実施した。Negative controlからのPCR偽陽性結果はどこからも無かったものの、若干の検査室においてPCRの低感度、誤ったsequencing結果の報告があり、是正を求めた。

2009年中のブラジル、中国、コロンビア、インド、ミャンマー、ベトナムにおける調査結果が、WHOのweekly epidemiological report 85: 281-284 (2010)に報告された。そのデータは今のところ耐性菌の割合は高くなく、dapsone耐性は12%、rifampicin耐性は8%、quinolone耐性は1%であった。コロンビア、ミャンマーにおいてやや高い耐性率が示された。我々が行った調査ではquinolone耐性はミャンマー、フィリピンからは耐性例が見いだされず、監視事業でも低い耐性菌出現率であったことから、quinoloneに対しては、いまだ耐性菌は問題とは考えられないが、今後その使用が普及することによりらい菌での耐性が増加することも予想される。本事業は監視拠点における耐性菌の経時的動向の調査を目的としており、今後の結果とともにそのMDTへの影響について注視していきたい。

研究と疾病対策の現場のかかわり

WHOによる本事業は、図らずも我々の基礎研究成果がGlobalなハンセン病対策に直接貢献している例となった。これまでにらい菌の基礎的細菌学あるいは分子生物学の成果がハンセン病対策の貢献した例としては古くはShepardによるマウスfootpadでのらい菌の増殖、PGL-1の抗原特異性、らい菌の全塩基配列の解明等々がある。これらは一見フィールドにおけるハンセン病対策と大きな隔たりがあるように思われるが、ハンセン病対策にその果たした役割は計り知れない。基礎研究者はややもするとこのような視点を失いがちであるが、疾病対策への貢献への道は遠くとも、最終的にはどのようなかたちでハンセン病に貢献できるのかを考えたうえでの研究が成されるべきであろう。その為には基礎研究者といえどもハンセン病対策の現場で何が問題となり、なにが求められているのかを絶えず注目していかなければならない。

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Our role in sentinel surveillance for drug resistance in leprosy by global leprosy programme

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Key words : drug resistance, leprosy, international cooperation, sentinel surveillance

Sentinel surveillance for drug resistance in leprosy by global leprosy programme has launched in 2006. Possible contribution of Japanese researchers to global leprosy control in the future were discussed on the base of circumstances of the project and our assignment in the sueveillance.

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チンパンジーとハンセン病

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〔受付・掲載決定：2010年9月9日〕

キーワード：チンパンジー、ハンセン病、野生動物、PGL-I、SNPs

はじめに

ハンセン病は、乳幼児期にらい菌［*Mycobacterium (M.)leprae*］に感染し、年余の潜伏期の後に発症すると推定されているが、不顕性感染を証明する方法が無いために、そのような仮説を検証することは出来なかった。今回、ヒトと最も近縁の霊長類として日本で肝炎ワクチン研究に用いられたチンパンジーに発症したハンセン病症例を経験した。ハンセン病に関する貴重な情報を提供する症例であると共に、実験動物の取り扱いや、チンパンジーとヒトとの関係などを含めて示唆に富む例であると考えられたので報告する。

1．チンパンジー・サンクチュアリ・宇土（CSU）について

有明海をはさんで島原の雲仙普賢岳と対峙する熊本県宇土半島の熊本県宇城市にある三和化学研究所内にThe Chimpanzee Sanctuary Uto (CSU) が2007年4月に誕生した。2007年8月から、京都大学と三和化学研究所は京都大学霊長類研究所に寄附研究部門「福祉長寿研究部門」を設置した（URL: http://cs-uto.org/）（図1）。かつて医学感染実験に使われたチンパンジーの余生を安寧に過ごさせるための施設である。

1978年以降、三和化学は東京大学医科学研究所、東京都立臨床医学研究所、化学及び血清療法研究所などからチンパンジーを引き取っていた。最後に引き取ったのが2000年の13人（チンパンジー、ゴリラ、オランウータンはヒトとともに4属でヒト科を構成する。チンパンジーの数え方は「人」、「頭」などがあるが、本稿では名前のあるチンパンジーなので「人」とした）であった。引き取った総数は約70人であった。引き取ったチンパンジーは繁殖目的で飼育されていたが、2000年以降7年間ほどは、チンパンジーを使って医学・薬学試験を受託していた。2006年医学・薬学試験をやめ、2007年にCSUと名称を変えた。CSUの敷地は約3.3ヘクタールに57人（2010年3月現在）のチンパンジーが生活している。半数は、アフリカから連れてこられた。残りの半数は、その子孫たちである。年齢は11歳から40歳までであり、年齢を1.5倍すると人間の年齢にほぼ相当する。

チンパンジーは1980年から約150人が日本に輸入されたと推定されている。主としてB型肝炎ワクチンの安全性試験に使用された[1,2]。当時、画

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期的であった第一世代B型肝炎ワクチン（ヒトキャリアの血漿由来）にB型肝炎ウイルスが残存する可能性を否定するためにはチンパンジーに接種することが不可欠だった。その後、非A非B型肝炎（C型肝炎）の研究にも使用されるようになった。現在は実験に使われているチンパンジーはいない。

ヒトとチンパンジーの遺伝的な違いは、DNAの塩基配列で約1.23%しかない[3]。そのため、ヒトが罹患するほぼすべての病気が両者で共通であり、感染症であれば双方向に伝染する。HIV感染症[4,5]やB型肝炎[6]、C型肝炎[7-9]など他に適当な実験動物が存在しない疾患もヒトとチンパンジーの共通感染症である。このように、人間にきわめて近いが故に感染実験の対象になってしまったことは、チンパンジーにとって悲劇であった。

2．チンパンジー　ハルナの出生地

CSUで生活しているチンパンジーの出生地はアフリカないし、日本であるが、今回の患者であるハルナについて述べる。

1980年ごろまで乳幼児のチンパンジーは先進国に高値で売れる商品だった。西アフリカ、シエラレオネ（Sierra Leone）では、森で狩り集められ、

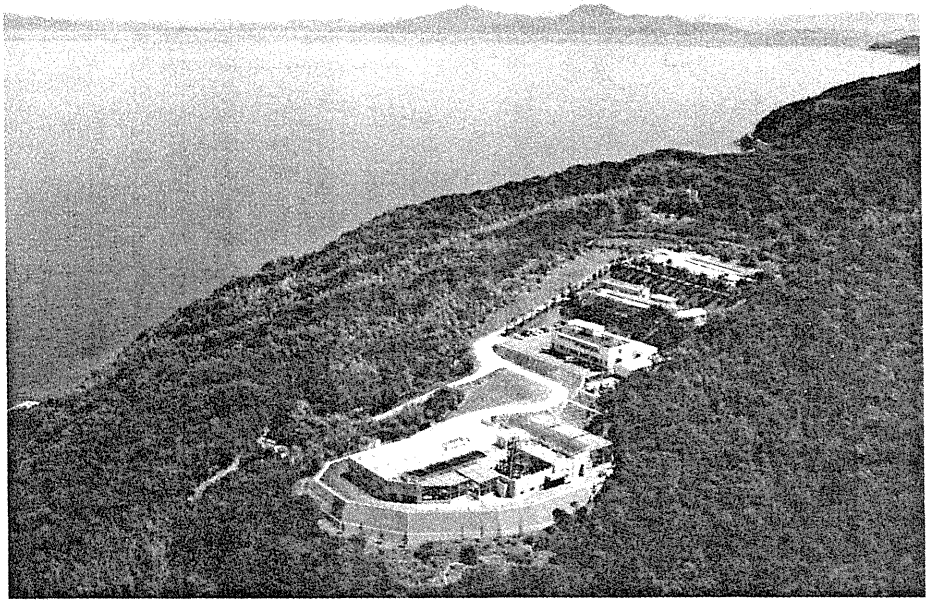

図1：チンパンジー・サンクチュアリ・宇土（CSU）、（全景航空写真）

図2：シエラレオネにあるチンパンジーの檻（現在は使われていない）
（樺沢氏提供）

小さな檻に入れられていた（図２）。親は散弾銃で射殺され肉として消費されていた。当時わが国には医学研究用として 151 人の乳幼児のチンパンジーが輸入されたが[2]、ハルナはそのうちの１人であった。

３．ハルナの生活歴とハンセン病を鑑別するまで

ハルナは女性チンパンジー（*Pan troglodytes verus*）で、1980 年３月（推定２歳）に日本に輸入されたが、体格から 1978 年出生と推定された（個体番号 58 番であったため、誕生日は 1978 年５月８日としている）。某大学でＢ型肝炎研究に使用された。1986 年６月（推定８歳）には某企業にてＣ型肝炎研究に使用された。2000 年３月（推定 21 歳）に実験から解放され CSU に移動し、その後は平穏に生活していた（図 3a）。

2009 年１月（推定 30 歳）、飼育者が顔面の腫脹、結節に気付いた。４月９日に「ハルナ左頬（鼻の横）腫れ」の報告があり、４月 15 日に観察したところ、眼瞼腫脹と口唇の変形を認めたが、食欲の変化などの異常を認めず、経過観察とした。５月に入ると、ハルナの顔の変形は顕著になった。明らかに異常と思える顔で、「獅子様顔貌」を連想させ、書物などに掲載されている「ハンセン病」の顔面に類似していた。他に鑑別する疾患として、菌状息肉症等いくつかリストアップし検討した。

４．ハルナの臨床所見および検査結果、診断

2009 年５月（推定 31 歳）に全麻下で全身精査、採血、皮膚スメアおよび生検を施行した（図 3b）。

体重 46.7kg。胸部Ｘ線、血算、生化学的所見など異常なし。血中の HBsAg、HBsAb、HBcAb、

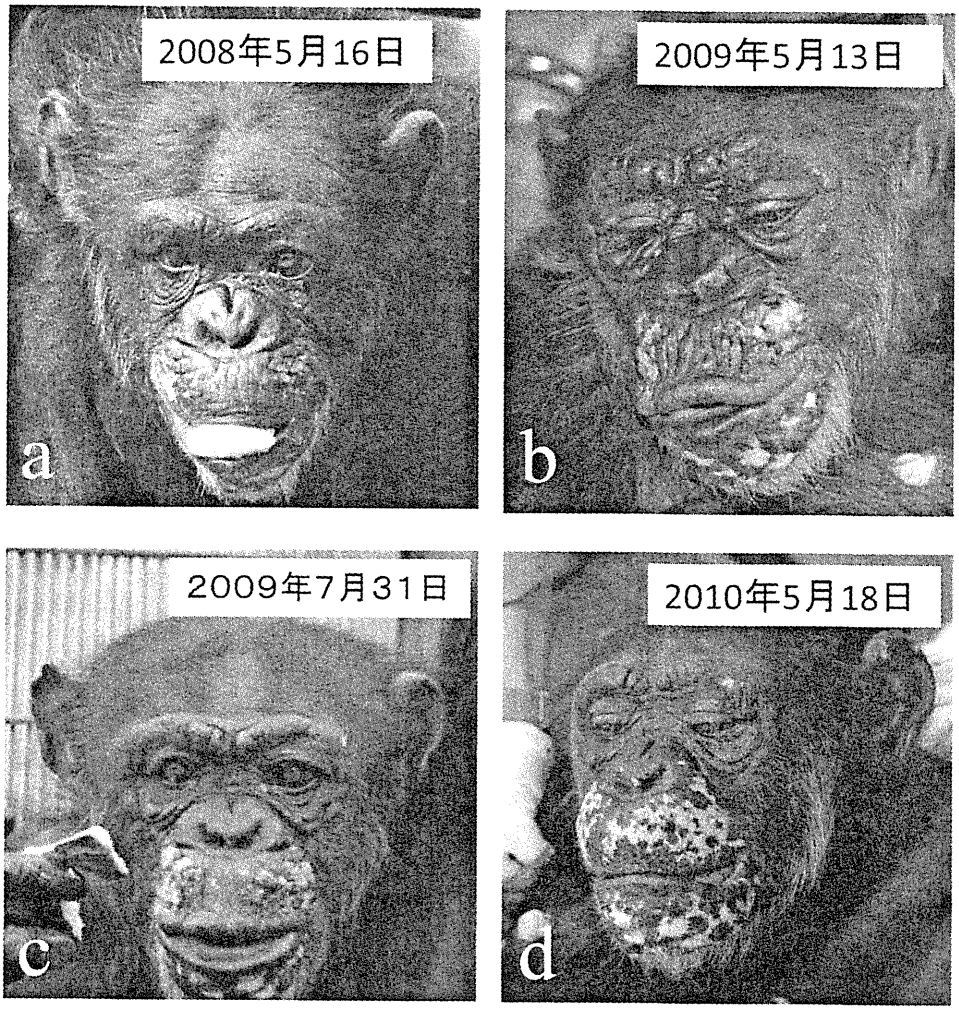

図３：ハルナの顔

a：2008 年５月 16 日（推定 30 歳）
b：2009 年５月 13 日（推定 31 歳）眼囲、鼻部、額部などに多数の結節を認める
c：2009 年７月 31 日（治療２ヶ月後）、結節は縮小している
d：2010 年５月 18 日（推定 32 歳）通常の顔に戻っている

HCV RNA 全て陰性。ツ反陰性。鼻腔スワブおよび左前腕部結節のスメア検査で抗酸菌を検出し、病理組織で泡沫細胞を多数認め、病理組織学的に LL 型ハンセン病と診断した（図 4）。皮膚組織を用いた結核菌 PCR は陰性。組織抽出 DNA を用いた PCR 検査によりらい菌 Hsp-70 陽性で、16S rRNA の塩基配列はらい菌 (*M.leprae*) DNA と 100% 一致した[10,11]。これらの所見から、ヒトに感染するものと同一の *M. leprae* による感染症、すなわちハンセン病と確定診断した。

5．治療および経過

2009 年 6 月 1 日より WHO/MDT/MB 開始。果物やジュースに混ぜるなどして投与し、2 ヶ月後には皮疹は改善し（図 3c）、5 ヶ月後鼻腔スワブは陰性化した。治療期間は WHO の推奨している 1 年間で終了した（図 3d）。

6．その他の検索結果

保存血清を用いた抗 PGL-I 抗体（セロディア® ・レプラ）は、2001 ～ 2004 年は陰性、2008 年偽陰性、発症後に陽転しており、治療開始半年後には再び陰性化した（図 5）[12-14]。2009 年の発症前に何らかの要因でらい菌が増殖しそれに伴って抗 PGL-I 抗体が陽転化し、治療によって菌が減少したことで陰性化したと考えられた。同時期に日本に輸入された同胞および同一ケージでの生活歴のある 13 人は全員陰性であった。また、鼻腔スワブの抗酸菌染色および PCR 検査でも菌は検出されなかった。また、全国の動物園などチンパンジーを飼育している施設に情報を提供し観察を依頼したが、ハルナ以外に皮膚症状からハンセン病を疑うチンパンジーはおらず、またサンクチュアリーの飼育者達にも知覚低下を伴う皮疹などの異常は認められなかった。ハルナのらい菌 DNA から Monot 等の報告にある 3 ヶ所の SNP の塩基配列を決定したところ、西アフリカに特徴的で日本には存在しない Type 4 であることが判明した[15,16]。このことから、ハルナは出生地である西アフリカで感染し、30 年近い潜伏期を経て、ハンセン病を発症したと判断した。

7．チンパンジーに発症したハンセン病　—世界の状況—

これまでにチンパンジーのハンセン病はアメリカから 3 例の報告があるのみで（表 1）[17-24]、本例が 4 例目である。何れも医学研究のためにアフリカから連れてこられた野生のチンパンジーで、4 例中 LL 型が 3 例、BL 型が 1 例であり、発症年齢も様々である。らい菌 DNA を証明し得たのは本例が初めてである。

8．ハルナが教えてくれた事

ハルナは 1980 年に来日してからは医学研究施設で生活しており、厳重な監視と健康管理が行われていたと考えられることから、日本でハンセン病に感染した可能性は除外できる。ハンセン病は乳幼児期に感染者との濃厚接触などにより多量の菌に曝露されることによって呼吸器感染し、長い潜伏期間の後に発症すると考えられてきたが[24]、ヒトの症例で実際の感染時期を特定することは困難であることから、この仮説を証明することは出来なかった。今回、ハルナの生活歴と SNP 解析の結果から、出生地である西アフリカ、シエラレオネで感染し、30 年近くの潜伏期を経て発症したものと断定することが出来た。

ハルナの感染経路としては、アフリカの野生チンパンジーにハンセン病が存在し乳幼児期に同胞

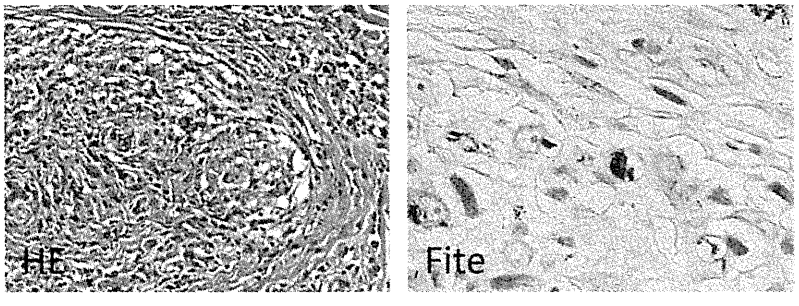

図 4：前腕部の皮膚の病理組織像
a：真皮に多数の泡沫細胞を認める（HE 染色、200X）
b：泡沫細胞には多数の抗酸菌を認める（Fite 染色、400X）

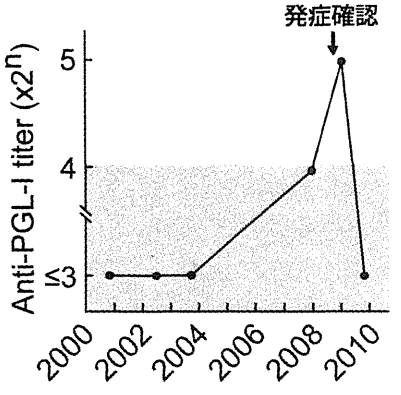

図 5：血清抗 PGL-I 抗体価の推移。ハンセン病発症時（2009 年 5 月）に一過性に陽性になった。