

ture rational attempts to cultivate *M. leprae* with notable advantages to our approach. Most importantly, our starting inoculum of *M. leprae* was freshly obtained for each experiment from infected nu/nu mice maintained under conditions designed to maximize *M. leprae* viability (42). We also were able to rapidly quantify the metabolic activity of *M. leprae* using the RR technique adapted by Franzblau (13). This assay can readily detect activity from as few as  $10^6$  bacilli with the results available in 1 wk (compared to 6 to 12 months when titrated in mouse footpads). RR data correlates well with other *in vitro* systems (13) but, more importantly, as shown in a recent series of 36 separate experiments, RR metabolic data correlated well with "viability" studied in the so-called "gold standard" mouse footpad system (42).

Whether intracellular or extracellular, *M. leprae* clearly prefers temperatures cooler than normal human body temperature, lending experimental credence to the clinical observations of generations of leprologists regarding the distribution of *M. leprae* in the cooler, permissive sites in human disease, the skin and mucous membranes of the upper respiratory tract. These studies also confirm and extend the results of dozens of other *in vitro* experiments (42) where 37°C appeared to be highly detrimental to *M. leprae* viability.

Under conditions of an effective cellular immune (CMI) response, MΦ are likely a major anti-microbial effector cell in host capacity to cope with the leprosy bacillus. We have previously shown that mouse MΦ, activated by interferon (IFN-γ), kill or inhibit a wide variety of intracellular pathogens (38), including *M. leprae* (32). Enhanced fusion of secondary lysosomes with *M. leprae*-containing phagosomes occurs in activated MΦ (39) and is accompanied by the enhanced production of reactive oxygen (ROI) and nitrogen intermediates (RNI) as potent anti-microbial mechanisms (2). We have subsequently shown *in vivo* in transgenic knock-out mice and *in vitro* in MΦ from these mice that ROI are relatively ineffective in comparison to the potent effects of RNI in host defense against *M. leprae* (1,3). However, although the enhanced production of RNI by activated MΦ is likely a principal antimicrobial mechanism, in each

of our studies, normal MΦ produced a measurable baseline level of RNI, insufficient to rapidly kill bacilli but perhaps sufficient to inhibit the long term viability of the fastidious, intracellular leprosy bacillus.

In choosing TGF and IL-10 as the cytokines that might bolster the intracellular survival of *M. leprae*, we were attempting to down regulate any innate ability of the normal MΦ to cope with the organism. TGF-β is produced by activated MΦ and other inflammatory cells and has a broad array of modulatory functions on the immune response. TGF-β has been shown to interfere with MΦ antimicrobial mechanisms including generation of ROI (43) and RNI (7), and has been shown to enhance the intracellular growth of *M. tuberculosis* in human monocytes (21). However, as employed in the present studies with mouse MΦ exogenous TGF-β had no detectable effect on sustaining intracellular *M. leprae* viability, a finding perhaps attributable to the enhanced innate antimicrobial ability of human monocytes in comparison to human monocyte derived MΦ (44) which are more akin to resident mouse peritoneal MΦ.

In contrast, supplementing media with IL-10 clearly affected the long term viability of *M. leprae* in mouse MΦ. IL-10 is produced in TH1 responses by T cells, B cells and MΦ (11,29). IL-10 has been shown to be a potent down-regulator of CMI to intracellular pathogens (33). *In vivo*, endogenous IL-10 dampened the CMI response to avirulent mycobacterial infection (35) and appeared to lead to loss of control of *M. tuberculosis* infection with widespread dissemination (9). IL-10 functions in part at the level of the macrophage by attenuating inducible nitric oxide synthase (iNOS) mRNA expression, iNOS activity and, by inference, NO production (22). *In vitro*, exogenous IL-10 inhibited production of nitric oxide (NO) in MΦ infected with *Babesia* merozoites (17). In our own studies, IL-10 did markedly inhibit the production of NO by IFN-activated MΦs but any inhibition by IL-10 of baseline production of NO by normal MΦs was below the limits of detection of the NO<sub>2</sub> assay (data not shown). Exogenous IL-10 also interferes with IFN-induced antimycobacterial MΦ activities as shown in studies with *M. bovis* (12).

In addition to sustained metabolism of *M.*

*leprae*, we also explored a morphological parameter of *M. leprae* vitality within cultured macrophages, bacillary elongation. Nakamura, *et al.* reported the elongation of *M. lepraemurium* in culture medium<sup>(28)</sup>, and in 1969 Chang and Anderson<sup>(4)</sup> evaluated intracellular growth of *M. lepraemurium* in cultured mouse peritoneal MΦ over a 12 to 17 week period and observed marked elongation of bacilli well before the appearance of bacillary multiplication. In our studies, in addition to sustained or enhanced metabolism, intracellular elongation of individual bacilli was observed after 4 weeks culture in murine MΦ maintained with IL-10. Elongation was first observed with the light microscope and subsequently confirmed with the transmission electron microscope. A drawback to the use of the TEM for such observations is that unlike light microscopy which revealed the bacilli at their full intracellular length, processing for the TEM required sectioning infected MΦ with no certainty that the full length of each bacillus was cut. Nevertheless sufficient numbers of clearly elongated bacilli were seen to confirm the light microscopy findings. Similarly, in preliminary studies with armadillo peripheral blood monocyte derived MΦ maintained at 33°C for 4 weeks, intracellular *M. leprae* were predominantly elongated (data not shown).

Septal formation has been described in TEM studies from human leprosy biopsies by Hirata<sup>(20)</sup> and was occasionally observed by us in individual bacilli under the TEM after 4 weeks of incubation of infected mouse MΦ in the presence of IL-10. The small sample size precluded quantification but septa appeared to be observed far less frequently in bacilli in the control MΦ. Septal formation in *M. leprae murium* in the mouse model has been reported to indicate dividing stage of the bacillus, and Hart and Rees<sup>(19)</sup> concluded that elongation *in vitro* was an inherent feature of *M. lepraemurium* that distinguishes it from *M. leprae* although it is likely that the *M. leprae* inocula employed were of very low viability. Experiments are currently underway to study *M. leprae* in our cell culture system employing an environmental scanning electron microscope which by passes the need for critical point drying and its attendant ar-

tifacts and will permit quantitation of elongation.

Further work with infected armadillo MΦ is also clearly warranted. Other than having a core temperature of ~33°C, little is known about the unique characteristics of *Dasybus novemcinctus*, the nine-banded armadillo, that render it as a permissive host for the leprosy bacillus<sup>(25)</sup>. *In vivo*, mononuclear phagocytes in virtually every organ of the natural or experimentally infected armadillo become heavily parasitized with propagating *M. leprae*<sup>(24)</sup>.

The inability to culture *M. leprae* has undoubtedly hindered almost every aspect of leprosy research, and thus, our understanding of this disease lags behind that of many others of bacterial etiology. These promising results represent only preliminary findings, but suggest that this approach of inhibiting the innate anti-microbial properties of the MΦ to bolster the intracellular survival of *M. leprae* may ultimately provide clues allowing the long sought-after cultivation of the leprosy bacillus. *In vitro* cultivation of *M. leprae* could make available for the first time, large quantities of pure bacilli produced inexpensively under defined conditions. Thus, large amounts of purified antigens would be available for basic and applied immunological studies, including the development of specific skin test antigens and vaccine preparations. The time and cost of screening new drugs and susceptibility testing of clinical isolates would also be greatly reduced. Our understanding of leprosy epidemiology might increase by determining the existence of human carriers, non-human reservoirs, or environmental sources of *M. leprae*. Phenotypic variation among cultured worldwide isolates could become feasible as might the generation and characterization of mutants. Finally, cultivation of *M. leprae*, in concert with the genome project, would clearly enhance our understanding of the physiology of this fastidious pathogen, including elucidation of metabolic pathways, studies of virulence mechanisms, drug resistance and the factors underlying "persistence."

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# Upregulation of T-Cell-Stimulating Activity of Mycobacteria-Infected Macrophages

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## Abstract

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Macrophages are one of the most abundant host cells to come in contact with mycobacteria. However, the infected macrophages less efficiently stimulate autologous T cells *in vitro*. We investigated the effect of the induction of phenotypic change of macrophages on the host cell activities by using *Mycobacterium leprae* as a pathogen. The treatment of macrophages with interferon- $\gamma$  (IFN- $\gamma$ ), GM-CSF and interleukin-4 deprived macrophages of CD14 antigen expression but instead provided them with CD1a, CD83 and enhanced CD86 antigen expression. These phenotypic features resembled those of monocyte-derived dendritic cells (DC). These macrophage-derived DC-like cells (MACDC) stimulated autologous CD4<sup>+</sup> and CD8<sup>+</sup> T cells when infected with *M. leprae*. Further enhancement of the antigen-presenting function and CD1a expression of macrophages was observed when treated with IFN- $\gamma$ . The *M. leprae*-infected and -treated macrophages expressed bacterial cell membrane-derived antigens on the surface and were efficiently cytolysed by the cell membrane antigen-specific CD8<sup>+</sup> cytotoxic T lymphocytes (CTL). These results suggest that the induction of phenotypic changes in macrophages can lead to the upregulation of host defence activity against *M. leprae*.

## Introduction

The extent of mycobacterial spread is closely associated with the disease severity and is controlled by bacterial antigen-specific cell-mediated immunity. The activation of CD4<sup>+</sup> and CD8<sup>+</sup> T-cell subsets is induced by cell-cell contact with antigen-presenting cells (APC), which is therefore the most critical host defence component against mycobacterial infection [1–3]. The mycobacteria reside in phagosomes of macrophages but restrict the capacity of the phagosomes to fuse with late endosomes/lysosomes [4] and avoid getting being processed. These observations suggest that mycobacteria-infected macrophage less efficiently express the bacterial antigens on their surface. Furthermore, macrophages generally lack the activity to load peptide derived from exogenous protein or bacterial protein to major histocompatibility complex (MHC) class I molecules. Therefore, it is assumed that macrophages are resistant to MHC-restricted-killing activities of cells such as CTL allowing mycobacteria to reside and hide in macrophages.

Here, we tried to upregulate the APC function of macrophages in order to enhance the host defence activity.

Leprosy, caused by *Mycobacterium leprae* infection, provides a useful model to evaluate immunoregulatory mechanism against an intracellular pathogen, because the disease extent is closely associated with the potential of host defence activity. *M. leprae* preferentially infects macrophages and Schwann cells [5–7] and induces clinical manifestation mainly in skin and peripheral nerves. However, the disease shows broad spectrum with the various skin manifestations from single lesion (tuberculoid leprosy) to almost entirely disseminated one (lepromatous leprosy). The mechanism that produces this broad spectrum involves the extent of cell-mediated immune response to *M. leprae*. We previously reported that macrophages phagocytosed *M. leprae*, but their antigens are not fully expressed on the surface of macrophages, which results in reduction or avoidance of their contact with T cells [8]. In addition to macrophages, monocyte-derived dendritic cells (DC) are well-characterized subset of professional APC, capable of stimulating both naïve and memory type autologous CD4<sup>+</sup> and CD8<sup>+</sup> T cells [9–11]. We also demonstrated that, in contrast to macrophages, the DC expressed *M. leprae*-derived antigens on the surface and stimulated

both T-cell subsets to produce interferon- $\gamma$  (IFN- $\gamma$ ) which is a representative type 1 cytokine and is considered to be associated with the killing of intracellular mycobacteria [2, 12]. Thus, DC can evoke the activation of type-1 T cells and control the multiplication of *M. leprae* [8, 13–15].

Macrophages are the most abundant primary cell type to come in contact with *M. leprae* and are frequently seen in inflamed tissues. These macrophages are thought to play a role in initial antimycobacterial immune responses for the better control of bacterial spread. Therefore, we tried to induce phenotypic changes in *M. leprae*-infected macrophages to DC-like cells and examined their antigen-presenting activities such as stimulation and differentiation of T cells into type-1 cells. Furthermore, we examined their susceptibility to killing activities of *M. leprae*-derived antigen-specific CTL.

## Materials and methods

**Preparation of cells and bacteria.** Peripheral blood was obtained under informed consent from healthy, but PPD-positive, individuals. Peripheral blood mononuclear cells (PBMC) were isolated using Ficoll–Paque Plus (Pharmacia, Uppsala, Sweden) and cryopreserved in liquid nitrogen until use, as previously described [16]. Macrophages were differentiated by culturing plastic adherent CD14<sup>+</sup> monocytes with RPMI 1640 medium containing M-CSF (R&D Systems, Minneapolis, MN, USA) [17]. For preparation of the monocytes, CD3<sup>+</sup> T cells were removed from either freshly isolated heparinized blood or cryopreserved PBMC using immunomagnetic beads coated with anti-CD3 monoclonal antibody (Dynabeads 450, Dynal, Oslo, Norway), and the plastic adherent cells were used as monocytes [16]. Monocyte-derived DC were differentiated from the monocytes [16, 18]. Briefly, the monocytes were cultured for 5 days in the presence of 50 ng of recombinant GM-CSF (Pepro Tech EC LTD, London, UK) and 10 ng of rIL-4 (Pepro Tech) per ml. rGM-CSF and rIL-4 were supplied every 2 days. Macrophage-derived DC (MACDC) were differentiated from macrophages as follows. Macrophages were treated with 300 U/ml of IFN- $\gamma$  during last 24 h of 3-day culture. On day 3, macrophages were washed, and the media were replaced with media containing rGM-CSF and rIL-4. The production of MACDC was conducted by using the same protocol to that of DC. In some cases, *M. leprae*-infected or -uninfected macrophages, MACDC and DC were further treated with indicated doses of maturation and activation factors for DC including CD40 ligand (L) (Pepro Tech) or lipopolysaccharide (LPS) (*Escherichia coli* 0111: B4, Difco Laboratories, Detroit, MI, USA).

*M. leprae* (Thai-53) was obtained from footpads of BALB/*c-nulnu* mice. The isolated bacteria were counted by Shepard's method [19] and were frozen at  $-80^{\circ}\text{C}$  until use. The viability of *M. leprae* was assessed by using

fluorescent diacetate/ethidium bromide test [20]. The macrophages and DC were infected with *M. leprae* by coculturing at indicated multiplicity of infection (MOI). The MOI was determined upon an assumption that all macrophages and DC were susceptible to infection with *M. leprae*. The macrophages and DC were previously shown to be similarly susceptible to the bacterial infection *in vitro* [8].

**Analysis of cell-surface and intracellular antigens.** The expression of cell-surface antigens on macrophages, MACDC and DC was analysed using FACScalibur (Becton Dickinson Immunocytometry System, San Jose, CA, USA). About  $1 \times 10^4$  live cells were analysed. For analysis of cell-surface antigens, the following monoclonal antibodies were used: FITC-conjugated monoclonal antibodies against HLA-ABC (G46-2.6, BD Biosciences, San Jose, CA, USA), HLA-DR (L243), CD14 (Leu-M3, Becton Dickinson) and CD86 (FUN-1, BD Biosciences) and phycoerythrin-labelled monoclonal antibodies against CD83 (HB15a, Immunotech, Marseille, France). Murine-unlabelled monoclonal antibody to CD1a (NA1/34, Serotec, Oxford, UK) was also used and was visualized by FITC-labelled goat F(ab')<sub>2</sub> antimouse immunoglobulin G (IgG) (Tago-immunologicals, Camarillo, CA, USA). Purified rabbit polyclonal antibodies (pAbs) to cell-wall proteins, cell membrane fractions and cytosol fractions of *M. leprae* each depleted of lipoarabinomannan (provided by Drs J. Spencer and P. J. Brennan, Colorado State University) were used. The details of the preparation are available at <http://www.cvmb.colostate.edu/mip/leprosy>. Among the *M. leprae*-derived fractions, membrane fraction was the most antigenic in terms of activation of adaptive immunity [21]. We also used pooled sera from 10 untreated lepromatous leprosy patients who were classified according to clinical criteria (WHO) based on skin smears (given by Dr H. Minagawa, Leprosy Research Center, Tokyo, Japan) [8]. FITC-conjugated murine antihuman Ig (Tago-immunologicals) was used as secondary antibody for detection. The optimal concentrations of monoclonal antibodies, pAbs and patient's pooled sera were determined in advance.

**Assessment of APC functions of macrophages, MACDC and DC.** The ability of macrophages, MACDC and DC infected with *M. leprae* to stimulate autologous T cells was assessed using an autologous stimulator T-cell-mixed reaction as previously described [8, 18]. The stimulators such as macrophages, MACDC and DC were treated with 50  $\mu\text{g}/\text{ml}$  of mitomycin C, washed extensively to remove extracellular bacteria and were used as a stimulator. CD4<sup>+</sup> and CD8<sup>+</sup> T cells purified using immunomagnetic beads coated with monoclonal antibodies to CD8 and CD4, respectively, were used as a responder population. Responder cells ( $1 \times 10^5$ /well) were plated in 96-well, round-bottom tissue culture plates, and stimulators were added to give an indicated stimulator responder T-cell

ratio. The T-cell proliferation during the last 10 h of a 4-day culture in the presence of 4% heat-inactivated human serum was quantified by incubating the cells with 1  $\mu\text{Ci}/\text{well}$  of [ $^3\text{H}$ ]-thymidine.

**Assessment of cytokine production.** Levels of the following cytokines were measured: IFN- $\gamma$  and IL-10 produced by CD4 $^+$  and CD8 $^+$  T cells stimulated for 4 days with macrophages, MACDC or DC and IL-12 p70 produced by *M. leprae*-infected or -uninfected macrophages and MACDC upon a stimulation with soluble form CD40L for 24 h. The concentrations of IFN- $\gamma$ , IL-12 p70 and IL-10 were quantified using OptEIA ELISA Set available from BD Biosciences.

**Assessment of cytotoxic activity of CD8 $^+$  T cells.** The susceptibility of *M. leprae*-infected macrophages and MACDC to the cytotoxic activity of CD8 $^+$  T cells was evaluated. Ten thousand macrophages and MACDC, either uninfected or infected with *M. leprae* at MOI 5, were used as a target cell. As an effector population, CD8 $^+$  CTL activated by stimulation with autologous DC, which were pulsed with 15  $\mu\text{g}/\text{ml}$  of *M. leprae*-derived cell membrane fractions, were used. The target cells were cocultured for 5 h with the effector cells at various effector/

target cell ratios. The supernatant was collected for lactate dehydrogenase (LDH) release assay. The concentration of LDH released by target cell death was measured according to the instructions of the assay kit (Cyto Tox 96 $^{\text{®}}$  Non-Radioactive Cytotoxicity Assay, Promega, Madison, WI, USA). The percent specific killing was calculated as follows:

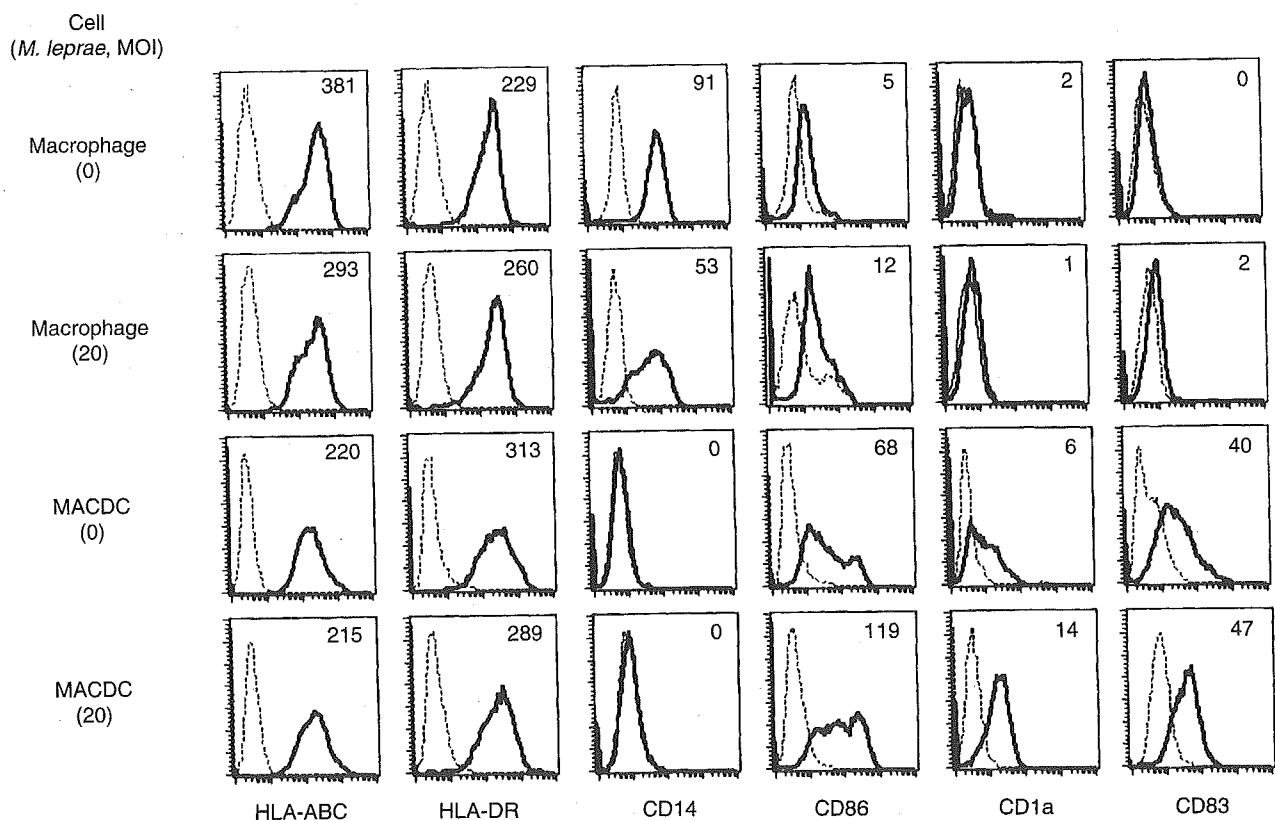
$$\frac{\text{LDH (test sample)} - \text{LDH (spontaneous effector cell level)} - \text{LDH (spontaneous target cell level)}}{\text{LDH (total target cell lysis level)} - \text{LDH (spontaneous target cell level)}}$$

**Statistical analysis.** Student's *t*-test was applied to demonstrate statistically significant differences.

## Results

### Upregulation of APC function of *M. leprae*-infected macrophages

Phenotypic characterization of MACDC was carried out by analyzing the surface expression of various molecules on macrophages and MACDC (Fig. 1). There were no



**Figure 1** Expression of various molecules on macrophages and macrophage-derived dendritic cell-like cells (MACDC) either uninfected or infected with *Mycobacterium leprae* [multiplicity of infection (MOI) 20]. Both macrophages and MACDC were infected with *M. leprae* for 5 days. The number represents the difference in mean fluorescence intensity between dotted and solid lines. A representative of three independent experiments is shown. Dotted line, control monoclonal antibody; solid line, monoclonal antibody.

apparent difference in the expression of HLA-ABC and HLA-DR antigens between uninfected macrophages and MACDC. Also, no significant upregulation of expression of these molecules was induced by *M. leprae* infection. While macrophages expressed CD14 antigen, the surface CD14 expression on MACDC was completely lost. On the contrary, the expression of CD86 antigen on MACDC was higher than that on macrophages. The expression of CD1a and CD83 antigens which is DC-specific marker was induced on MACDC, both of which level was further upregulated by *M. leprae* infection. These results indicated that MACDC showed phenotypic characteristics similar to monocyte-derived DC, and MACDC were at least partially activated by *M. leprae* infection. Further characterization of MACDC was conducted by comparing

the APC function of MACDC with that of monocyte-derived DC (Table 1). Both DC and MACDC induced proliferation of autologous CD4<sup>+</sup> and CD8<sup>+</sup> T cells in a bacterial dose-dependent manner, but a higher T-cell proliferation was induced by MACDC rather than DC. More than 75% of the MACDC-induced T-cell proliferation was suppressed by monoclonal antibody to MHC or CD86 antigens (data not shown). Previously, we showed that monocyte-derived DC were resistant to exogenous factors such as LPS and CD40L in terms of APC function [8], but using MACDC, both factors upregulated the T-cell-stimulating function of *M. leprae*-infected, but not uninfected, MACDC (Table 2). The proliferation of both CD4<sup>+</sup> and CD8<sup>+</sup> T-cell subsets were upregulated in a manner dependent on the dose of *M. leprae*.

Table 1 Proliferative response of autologous T cells to *Mycobacterium leprae*-infected macrophage-derived dendritic cell (DC)-like cells (MACDC)\*

<i>M. leprae</i> (MOI)	Stimulator T/DC	CD4		CD8	
		20	40†	10†	20
0	DC	1.9 ± 0.6	1.6 ± 0.5	1.8 ± 0.4	1.5 ± 0.3
	MACDC	2.1 ± 0.6	1.7 ± 0.5	1.7 ± 0.4	1.3 ± 0.2
5	DC	3.8 ± 0.9‡	2.7 ± 0.9‡	3.7 ± 1.2‡	2.6 ± 0.7‡
	MACDC	14.6 ± 2.3‡	8.0 ± 2.0‡	13.9 ± 3.3‡	10.6 ± 2.8‡
20	DC	6.9 ± 1.2‡	7.6 ± 1.3‡	10.1 ± 2.1§	8.1 ± 2.0‡
	MACDC	22.3 ± 5.2‡	17.9 ± 5.0‡	21.6 ± 4.1§	18.2 ± 4.3‡

\*The responder CD4<sup>+</sup> and CD8<sup>+</sup> T cells (1 × 10<sup>5</sup>/well) were stimulated for 4 days with an indicated dose of autologous monocyte-derived DC (DC) or MACDC. MACDC were differentiated from macrophages, either uninfected or infected for 5 days with an indicated dose of *M. leprae*, by using IFN-γ, rGM-CSF and rIL-4. The proliferation of responder cells was quantified by an incorporation of [<sup>3</sup>H]-thymidine. Representative of three separate experiments is shown. Assays were done in triplicate, and results are expressed as mean ± SD.

†[<sup>3</sup>H]-thymidine uptake (×10<sup>3</sup> cpm).

‡*P* < 0.05.

§*P* < 0.01.

Table 2 Effect of exogenous factors on the antigen-presenting cell function of macrophage-derived dendritic cell (DC)-like cells (MACDC)\*

<i>M. leprae</i> (MOI)	Tested factor T/DC	CD4		CD8	
		20	40†	10†	20
0	None	1.0 ± 0.0	0.8 ± 0.0	1.3 ± 0.2	0.9 ± 0.1
	LPS	1.5 ± 0.3	1.2 ± 0.2	1.7 ± 0.3	1.2 ± 0.2
	CD40L	2.8 ± 0.2	1.9 ± 0.2	2.3 ± 0.4	1.9 ± 0.3
5	None	5.7 ± 0.8‡	4.5 ± 0.7‡**	4.9 ± 0.4¶	3.9 ± 0.3‡§
	LPS	16.5 ± 2.0‡	11.7 ± 2.0**	13.8 ± 0.7¶	10.9 ± 0.9‡
	CD40L	11.2 ± 0.3‡	8.3 ± 0.4‡	10.4 ± 0.7¶	7.6 ± 0.8§
20	None	9.3 ± 1.2‡	6.9 ± 0.8‡§	7.7 ± 0.6‡¶	6.4 ± 0.6‡¶
	LPS	23.3 ± 2.4‡	15.7 ± 2.1§	21.7 ± 2.0‡	16.3 ± 1.1¶
	CD40L	16.5 ± 0.5‡	12.3 ± 0.5‡	15.0 ± 0.8¶	11.9 ± 1.0‡

\*The responder CD4<sup>+</sup> and CD8<sup>+</sup> T cells (1 × 10<sup>5</sup>/well) were stimulated for 4 days with autologous MACDC. MACDC were differentiated from macrophages, either uninfected or infected, for 5 days with an indicated dose of *Mycobacterium leprae*, by using IFN-γ, rGM-CSF and rIL-4 and were further treated with exogenous factors including LPS (30 ng/ml) and soluble form of CD40L (1 μg/ml). The proliferation of responder cells was measured by an incorporation of [<sup>3</sup>H]-thymidine. Representative of three separate experiments is shown. Assays were done in triplicate, and results are expressed as mean ± SD.

†[<sup>3</sup>H]-thymidine uptake (×10<sup>3</sup> cpm).

‡*P* < 0.005.

§*P* < 0.01.

¶*P* < 0.001.

\*\**P* < 0.05.



Next, the role of exogenous IFN- $\gamma$  on macrophages and its effect on MACDC production was determined (Fig. 2). While no apparent alterations were induced by 24 h IFN- $\gamma$  treatment in the expression of HLA-ABC and HLA-DR antigens (data not shown), the expression of CD1a was significantly upregulated by IFN- $\gamma$  treatment or by *M. leprae* infection. The highest CD1a expression on macrophages was achieved when both *M. leprae* infection and IFN- $\gamma$  treatment were conducted. Similar upregulation was observed in the expression of CD86 antigen. Moreover, when the IFN- $\gamma$  treatment was assessed from the functional aspect, it upregulated APC function of *M. leprae*-infected MACDC (Table 3). The IFN- $\gamma$  treatment on macrophages significantly upregulated activities of MACDC to stimulate both CD4<sup>+</sup> and CD8<sup>+</sup> T-cell subsets, and 300 U/ml of IFN- $\gamma$  provided the optimal upregulation. However, a sole IFN- $\gamma$  treatment was not sufficient and both GM-CSF and IL-4 were required for upregulating the APC function of macrophages (data not shown).

Then, we examined whether MACDC activated type-1 T cells by measuring IFN- $\gamma$  production by T cells (Table 4). While both the bacteria-infected and -uninfected macrophages, even after being stimulated with an exogenous IFN- $\gamma$ , did not stimulate autologous CD4<sup>+</sup> and CD8<sup>+</sup> T cells, MACDC derived from *M. leprae*-infected macrophages did stimulate both subsets of T cells to produce IFN- $\gamma$ . However, neither IL-4 nor IL-10 was produced by T cells (data not shown). The IFN- $\gamma$  production by T cells stimulated with MACDC exhibited a bell-shape phenomenon. When up to MOI 20 of *M. leprae* was infected to precursor macrophages, T cells were activated in a bacterial dose-dependent manner, but when more than MOI 20 of the bacteria was infected, the APC function of MACDC decreased. Similar T-cell activation pattern was also observed with heat-killed *M. leprae* (data not shown). Furthermore, MACDC attained an ability to

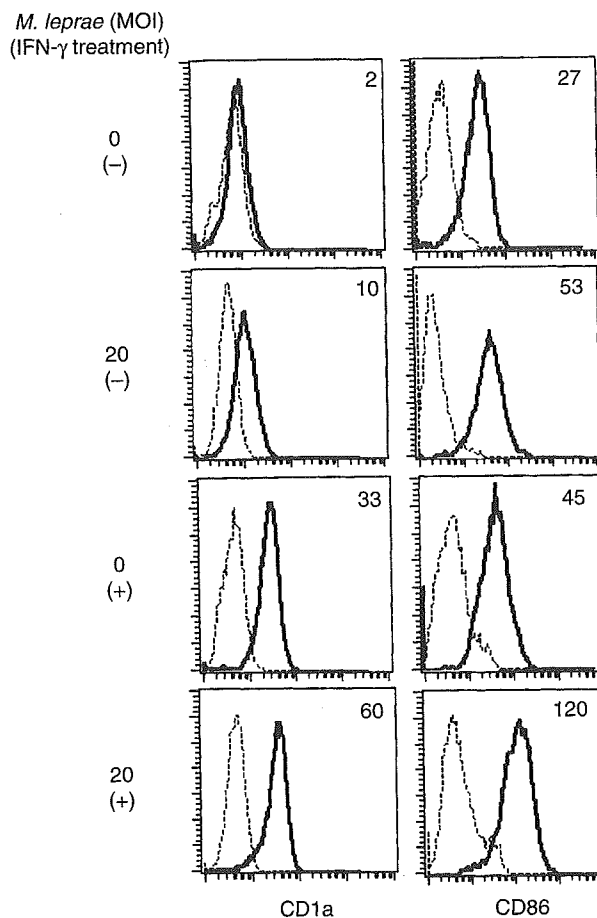


Figure 2 Expression of CD1a and CD86 antigens on macrophages. Macrophages were uninfected or infected with *Mycobacterium leprae* [multiplicity of infection (MOI) 20] on day 2 of culture and were subsequently treated with IFN- $\gamma$  (300 U/ml) for 24 h on day 3 of culture. The number represents the difference in mean fluorescence intensity between dotted and solid lines. A representative of three independent experiments is shown. Dotted line, control monoclonal antibody; solid line, monoclonal antibody.

Table 3 Effect of exogenous IFN- $\gamma$  on the T-cell-stimulating activity of macrophage-derived dendritic cell (DC)-like cells (MACDC)\*

<i>M. leprae</i> (MOI)	IFN- $\gamma$ (U/ml) T/DC	CD4		CD8	
		20	40†	10†	20
0	0	4.7 ± 1.2	3.2 ± 1.1	4.9 ± 2.0	3.6 ± 1.8
20	0	15.5 ± 3.0‡	11.5 ± 2.1§	14.9 ± 1.9‡§	11.4 ± 1.6§
20	100	25.7 ± 2.8‡	18.9 ± 2.7§	23.2 ± 2.2‡	18.4 ± 2.4§
20	300	33.6 ± 2.9‡	26.1 ± 3.0§	32.7 ± 3.0§	26.3 ± 3.0§
20	1000	11.1 ± 4.5	0.9 ± 0.6	11.9 ± 1.6	8.8 ± 0.7

\*The responder CD4<sup>+</sup> and CD8<sup>+</sup> T cells ( $1 \times 10^5$ /well) were stimulated for 4 days with an indicated dose of autologous MACDC. MACDC were produced from macrophages, either uninfected or infected with *Mycobacterium leprae* for 5 days at multiplicity of infection (MOI) 20 and treated with an indicated dose of IFN- $\gamma$ . The proliferation of responder cells was measured by an incorporation of [<sup>3</sup>H]-thymidine. Representative of three separate experiments is shown. Assays were done in triplicate, and results are expressed as mean ± SD.

†[<sup>3</sup>H]-thymidine uptake ( $\times 10^3$  cpm).

‡ $P < 0.001$ .

§ $P < 0.005$ .

**Table 4** Production of IFN- $\gamma$  by stimulated T cells and IL-12 p70 by *Mycobacterium leprae*-infected macrophage-derived dendritic cell (DC)-like cells (MACDC)\*

Stimulator	M. leprae (MOI) T/DC	CD4		CD8		IL-12 $\uparrow$ (pg/ml)
		20	40 $\ddagger$	10 $\ddagger$	20	
M $\emptyset$	0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.1 $\pm$ 0.1	0.1 $\pm$ 0.0	4.6 $\pm$ 0.9
	5	0.2 $\pm$ 0.0	0.1 $\pm$ 0.0	0.2 $\pm$ 0.0	0.3 $\pm$ 0.0	3.0 $\pm$ 1.0
	20	0.1 $\pm$ 0.0	0.1 $\pm$ 0.1	0.3 $\pm$ 0.1	0.1 $\pm$ 0.1	0.2 $\pm$ 0.0
MACDC	0	6.2 $\pm$ 0.8 $\S$	3.1 $\pm$ 0.7 $\S\parallel$	5.4 $\pm$ 1.0 $\S$	2.9 $\pm$ 0.8 $\S^{**}$	11.4 $\pm$ 9.1 $\parallel^{**}$
	5	73.0 $\pm$ 2.3 $\S$	22.6 $\pm$ 0.8 $\parallel$	34.3 $\pm$ 2.1 $\S$	11.1 $\pm$ 1.0 $\S$	43.6 $\pm$ 7.4 $\S^{**}$
	20	173.5 $\pm$ 6.9 $\S$	82.3 $\pm$ 2.1 $\S$	84.9 $\pm$ 3.1 $\S$	31.1 $\pm$ 1.9 $\S^{**}$	56.1 $\pm$ 8.8 $\parallel$
	80	19.2 $\pm$ 1.4	9.5 $\pm$ 1.0	15.6 $\pm$ 0.9	7.7 $\pm$ 0.9	Nor detected

\*The responder CD4 $^{+}$  and CD8 $^{+}$  T cells ( $1 \times 10^5$ /well) were stimulated for 4 days with an indicated dose of autologous macrophages or MACDC. MACDC were differentiated from macrophages, either uninfected or infected for 5 days with an indicated dose of *M. leprae*, by using IFN- $\gamma$ , rGM-CSF and rIL-4. The concentration of IFN- $\gamma$  produced by stimulated T cells was measured by ELISA.

$\ddagger$ Macrophages and MACDC ( $2 \times 10^5$ /well) were stimulated in the presence of CD40L for 24 h and the concentration of IL-12 p70 was measured. Representative of three separate experiments (IFN- $\gamma$  and IL-12 production) is shown. Assays were done in triplicate, and results are expressed as mean  $\pm$  SD.

$\parallel$ IFN- $\gamma$  (pg/ml).

$\S$  $P < 0.0005$ .

$\parallel$  $P < 0.0001$ .

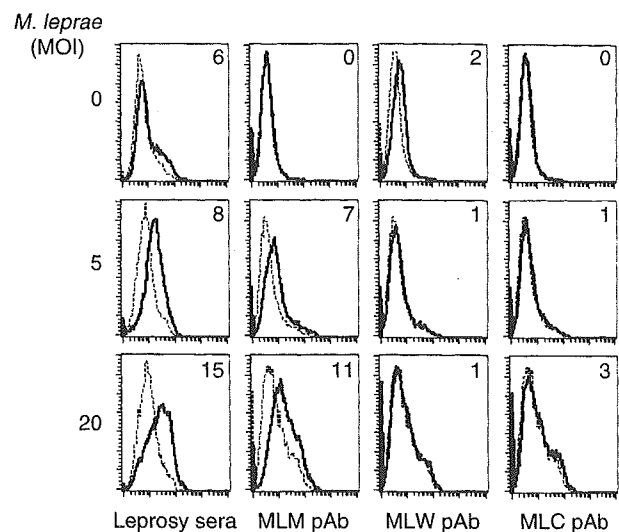
$\S\S$  $P < 0.001$ .

produce IL-12 p70 by stimulation with CD40L. The infection of MACDC with *M. leprae* further upregulated the cytokine production. However, macrophages did not produce the cytokine by any stimuli such as CD40L or *M. leprae* infection.

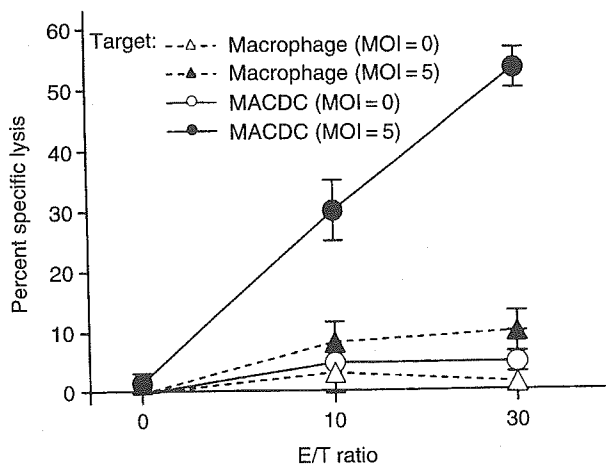
#### Susceptibility of MACDC to CTL-killing activity

We examined the expression of *M. leprae*-derived antigens on the MACDC by using leprosy patient sera and pAbs to subcellular components of the bacteria, as it has been reported that leprosy sera detected *M. leprae*-derived antigens on the bacteria-infected DC [8]. *M. leprae*-infected MACDC expressed molecules which reacted with sera obtained from leprosy patients (Fig. 3). Furthermore, these mycobacteria-infected MACDC were also positively stained with pAb to cell membrane fraction of *M. leprae* but did not react to pAbs against cell wall or cytosol fractions. These results may suggest that MACDC expressed cell membrane components on the surface. In order to clarify the significance of expression of membrane components, we assessed whether *M. leprae*-infected MACDC could be more efficiently killed by *M. leprae* cell membrane-specific CTL than the bacteria-infected macrophages (Fig. 4). In a previous report, we showed that CD8 $^{+}$  T cells, stimulated *in vitro* with *M. leprae*-derived cell membrane fraction-pulsed DC, produced intracellular perforin [21]. In this experiment, we used these perforin-producing CD8 $^{+}$  T cells as an effector population. While CD8 $^{+}$  T cells stimulated with DC unpulsed with any specific antigens did not kill either macrophages or MACDC regardless of

the bacterial infection (data not shown), CD8 $^{+}$  T cells stimulated with cell membrane-pulsed DC did kill *M. leprae*-infected, but not uninfected, target cells. More than 50% of *M. leprae*-infected MACDC were killed



**Figure 3** Expression of *Mycobacterium leprae*-derived molecules on macrophage-derived dendritic cell-like cells (MACDC). MACDC were differentiated from macrophages either uninfected or infected with an indicated dose of *M. leprae* for 5 days and were stained with lepromatous leprosy patients' sera and polyclonal antibody (pAb) to *M. leprae* subcellular fractions. The number represents the difference in mean fluorescence intensity between dotted and solid lines. A representative of three independent experiments is shown. MLC, *M. leprae* cytosol fraction; MLM, *M. leprae* cell membrane fraction; MLW, *M. leprae* cell-wall fraction. Dotted line, normal rabbit immunoglobulin G; solid line, pAb.



**Figure 4** Susceptibility of *Mycobacterium leprae*-infected macrophage-derived dendritic cell-like cells (MACDC) to *M. leprae*-derived membrane antigen-specific CTL. *M. leprae*-derived membrane antigen-specific CD8<sup>+</sup> CTL were differentiated from CD8<sup>+</sup> T cells by stimulation with the antigen-pulsed autologous mature DC as described previously [21] and were used as effector cells. Macrophages and MACDC either uninfected or infected with *M. leprae* (MOI 5) for 5 days were cocultured with effector cells for 5 h at the indicated E/T ratio. Lactate dehydrogenase released by cells were measured. The mean  $\pm$  SD of triplicate assay and a representative of three independent experiments is shown.

at the E/T ratio of 30, and these MACDC were more efficiently killed than the bacteria-infected macrophages.

## Discussion

We tried to induce phenotypic change in mycobacteria-infected macrophages and examined its effect on the host defence activity such as antigen-presenting capacity and the sensitivity to CD8<sup>+</sup> CTL. It is known that *M. leprae*-infected macrophages produce IL-10 and stimulate T cell less efficiently [22], and in our hands too, the infected macrophages did not stimulate T cells vigorously, even after being treated with exogenous IFN- $\gamma$  (Table 4). We have previously demonstrated that monocyte-derived DC also exhibited similar *in vitro* susceptibility to *M. leprae* infection, but in contrast to macrophages, the DC stimulated autologous T cells [8]. However, when the DC were infected with a low number of bacteria, they did not vigorously stimulate T cells, and additional stimulation by bacterial subcellular components was necessary for induction of significant T-cell activation [21]. The exact reason for the less efficient T-cell-activating ability of *M. leprae* is not fully uncovered but might be due to scarcity of antigens on the surface of *M. leprae* as a consequence of large number of pseudogenes in their genome [23]. On the other hand, from the aspect of host defence, these observations indicate the necessity to recruit professional APC,

which can initiate T-cell responses by responding to a small number of the bacteria.

In animal models, *M. tuberculosis* induces disease similar to human tuberculosis [24–27], in which the bacteria infect both macrophages and DC [1, 14, 25, 28]. Although these APC are found in the lesion microenvironment, macrophages and DC seem to respond differently following infection. Macrophages produce IL-10 upon an infection with the mycobacterium, and the secreted IL-10 lead naïve T cells to unresponsiveness against the bacterial antigens, although they promote the formation of tuberculous granuloma by residing in close opposition with activated T cells [29]. However, they produce no detectable level of IL-12. In contrast to macrophages, DC initiate both type-1 CD4<sup>+</sup> and CD8<sup>+</sup> T-cell activation and further act as a primary producer of IL-12 p70 following mycobacterial infection. The DC-mediated IL-12 triggers rapid differentiation of both T-cell subsets into type-1 T cells which cognately interact with DC. These T cells produce IFN- $\gamma$ , which in turn, contributes to induce mycobacteriocidal action to APC such as macrophages [14, 29, 30]. Considering these facts, we tried to induce phenotypic changes in mycobacteria-infected macrophages, so that T cells could be stimulated. On treatment with rIFN- $\gamma$ , rGM-CSF and rIL-4, *M. leprae*-infected macrophages were phenotypically transformed to DC-like cells (MACDC). These cells expressed CD1a and CD83 antigens but lacked the expression of CD14 and produced IL-12 and induced responses, such as proliferation and IFN- $\gamma$  production, of both CD4<sup>+</sup> and CD8<sup>+</sup> T-cell subsets. These results are partly supported by previous report that Th1-polarizing potential was observed when macrophages, not infected with any bacteria, were treated with GM-CSF and IL-4 [31]. Although, monocyte-derived DC required higher dose of bacterial infection for T-cell activation, MACDC showed a distinctive feature, in that they initiated T-cell proliferation more efficiently than the DC with rather small number of *M. leprae* (Table 1). Therefore, it may be reasoned out that MACDC and DC contribute distinctively to the host defence; the former might be more important in the microenvironment where a small number of *M. leprae* exist. This hypothesis might be associated with the previous finding that CD1a<sup>+</sup> and CD83<sup>+</sup> cells were enrolled in tuberculoid leprosy lesion [22], although it was not clarified whether they originated from macrophages or monocytes.

IFN- $\gamma$  contributed to the efficient development of MACDC by upregulating the expression of CD1a and CD86 molecules on macrophages (Fig. 2). In murine system, IFN- $\gamma$  is known to be associated with production of the reactive nitrogen intermediates which can directly kill intracellular mycobacteria [32]. Although there is no definite evidence suggesting the association of such intermediate products with mycobacterial killing in human, IFN- $\gamma$  can activate macrophages and kill the intracellular

bacteria. In this study, we showed that IFN- $\gamma$  did not directly endow macrophages with T-cell-stimulating activity but contribute to efficient differentiation of macrophages to MACDC by upregulating the expression of CD86 and CD1a molecules (Fig. 2). Furthermore, the IFN- $\gamma$  obviously upregulated T-cell-activating ability of MACDC (Table 3).

Another peculiar feature of *M. leprae*-infected MACDC is that they showed an enhanced susceptibility to killing activity of *M. leprae* cell membrane antigen-specific CD8<sup>+</sup> CTL, although the identification of immunodominant antigenic determinants remains unknown. When compared to *M. leprae*-infected macrophages, the *M. leprae*-infected MACDC were more efficiently killed by CTL. It is interesting to note that *M. leprae*-infected MACDC expressed antigens reactive to pAb to cell membrane components, but not antigens recognized by pAb to cell wall or cytosol components. In addition, our previous data showed that cell membrane was the most efficient antigen among *M. leprae* subcellular components for the activation of CD8<sup>+</sup> CTL [21]. When we compared *M. leprae*-infected MACDC and the macrophages, there were no difference in the expression of MHC class I and class II antigens, but only the former expressed *M. leprae*-derived antigens on the surface. Therefore, the expression of cell membrane antigens on MACDC might be closely associated with the enhanced susceptibility to killing activity.

Taken together, an induction of phenotypic change on *M. leprae*-infected macrophages resulted in enhanced type-1 T-cell-stimulating ability and an upregulated susceptibility to CTL activity. These observations may be further useful for developing immunotherapeutic tools against intracellular pathogens which threaten humans worldwide.

## Acknowledgments

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## DETECTION OF DAPSONE RESISTANT MYCOBACTERIUM LEPRAE BY DNA SEQUENCE ANALYSIS FROM A TURKISH RELAPSED LEPROSY PATIENT

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### SUMMARY

*Mycobacterium leprae* obtained from the relapsed case after 43-year dapsone monotherapy was submitted to sequence analysis of the genes associated with resistance to antileprosy drugs for drug susceptibility testing. Single nucleotide substitution was revealed at the codon 55 of the *folP* gene, which had been reported to confer resistance to dapsone. No mutations in the *rpoB* and *gyrA* genes indicated this isolate was susceptible to rifampicin and fluoroquinolones respectively. Drug susceptibility was conducted easily and rapidly by the sequence analysis compared with that by mouse footpad method.

**Key words:** Leprosy, *Mycobacterium leprae*, Drug resistance, Dapsone

### INTRODUCTION

Many cases of relapse have been observed among the patients treated by dapsone monotherapy (11) or multidrug therapy (5). High prevalence of drug resistance was also indicated in the relapsed cases from the patients previously treated by single drug therapy (3, 7). Clarification of the susceptibility to antileprosy drugs enhances the effective treatment. Drug susceptibility test has been conducted by mouse footpad method for many years but hampered by the cumbersome nature of the methods (10). Mutations of 1 genes associated with drug resistance to antileprosy drugs were revealed and confirmed (1,4,6,12). These advances enabled us to examine the susceptibility to antileprosy drugs by sequence analysis especially in the countries where the examination with mouse footpad test is not feasible.

We report here the drug resistance profile of *M. leprae* obtained from the relapsed case with a prolonged dapsone monotherapy by the sequence analysis for the genes relevant

to dapsone, rifampicin and quinolones resistance.

### MATERIALS and METHODS

**Patient:** A slit skin sample was collected from a 60-year old Turkish man, who was first diagnosed with leprosy at the age of 15, by the same method for BI test. The patient had been treated with DDS monotherapy for 43 years until the year 2000 and he faced no clinical health problem during the time. After stopping the administration, he had erythematous maculae in the face, extremities and trunk. The BI was +5 in skin lesion when sample was collected in 2002, however, no data of BI test during dapsone monotherapy was available. Slit skin smear sample on the blade was soaked in 70% ethanol until further analysis.

**Preparation of template and sequence analysis:** Bacilli on the surface of the blade were removed and suspended in 70% ethanol followed by washing with PBS. Genomic DNA

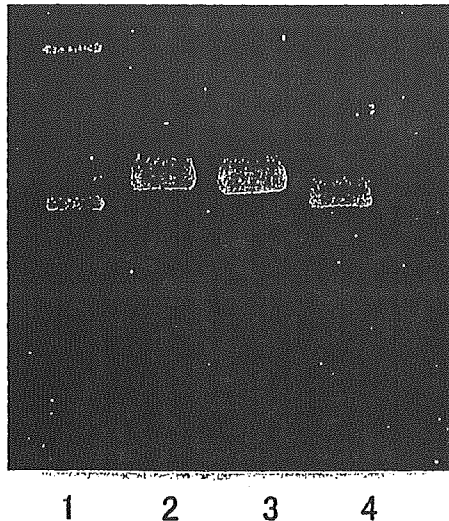
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Figure 1. PCR products for sequencing of genes. Lane 1, 50bp ladder DNA marker. Lane 2, *folP1* (390bp). Lane 3, *rpoB* (381bp). Lane 4, *gyrA* (350bp).



was prepared as described previously (2). DNA fragments were amplified by G mixture of FailSafe PCR System (EPICENTRE, Madison, WI, USA) that corresponded to the regions of the *rpoB* (forward primer: 5'-CAGGACGTCGACGCGATCAC-3', reverse primer: 5'-CAGCGGTCAAGTATTCGATC-3'), *folP1* (forward primer: 5'-GCTTCTCGTGCCGAAGCGCTC-3', reverse primer: 5'-GCCATCGCGG-GATCTGCTCGCCA-3') and *gyrA* (5'-for-

ward primer: CAGGTGACGGTTCTATACAG-3', reverse primer: 5'-TACCCGGC-GAACCGAAATTG-3') genes associated with resistance to rifampicin, dapsone and fluoroquinolones, respectively. The PCR products were sequenced in the same manner as previous methods (7,9), however, DNA samples were recovered by MinElute Gel Extraction Kit (QIAGEN, GmbH, Germany) instead of EASYTRAP (Takara, Siga, Japan) after electrophoresis of PCR products.

## RESULTS

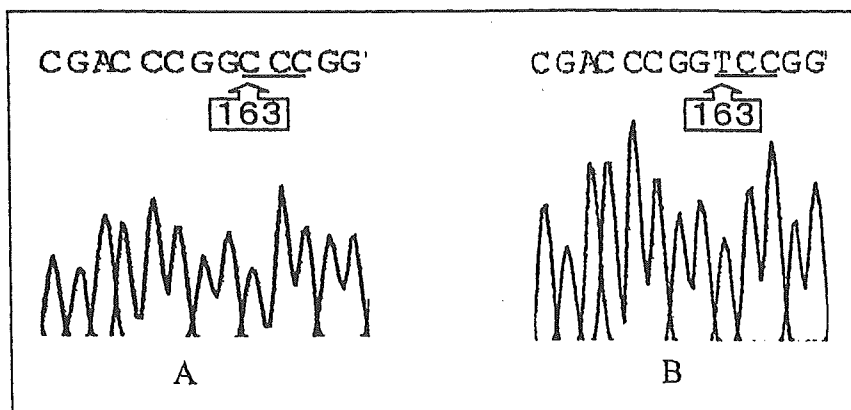
DNA fragments of the target regions of the *folP1*, *rpoB* and *gyrA* genes were amplified and electrophoresed on an agarose gel. The PCR product of the expected length was observed by ethidium bromide staining and UV irradiation (Figure 1). Sequence analysis revealed a single mutation in the *folP1* gene at codon 55 (CCC→TCC) resulting in the substitution of a proline for a serine (Figure 2). The results suggested that the clinical isolate was resistant to dapsone. No mutations of amino acids in *rpoB* at 513, 516, 526, 531 and 533, and amino acids in *gyrA* at 89 and 91 were detected. The results indicated that this isolate was sensitive to rifampicin and fluoroquinolones respectively.

## DISCUSSION

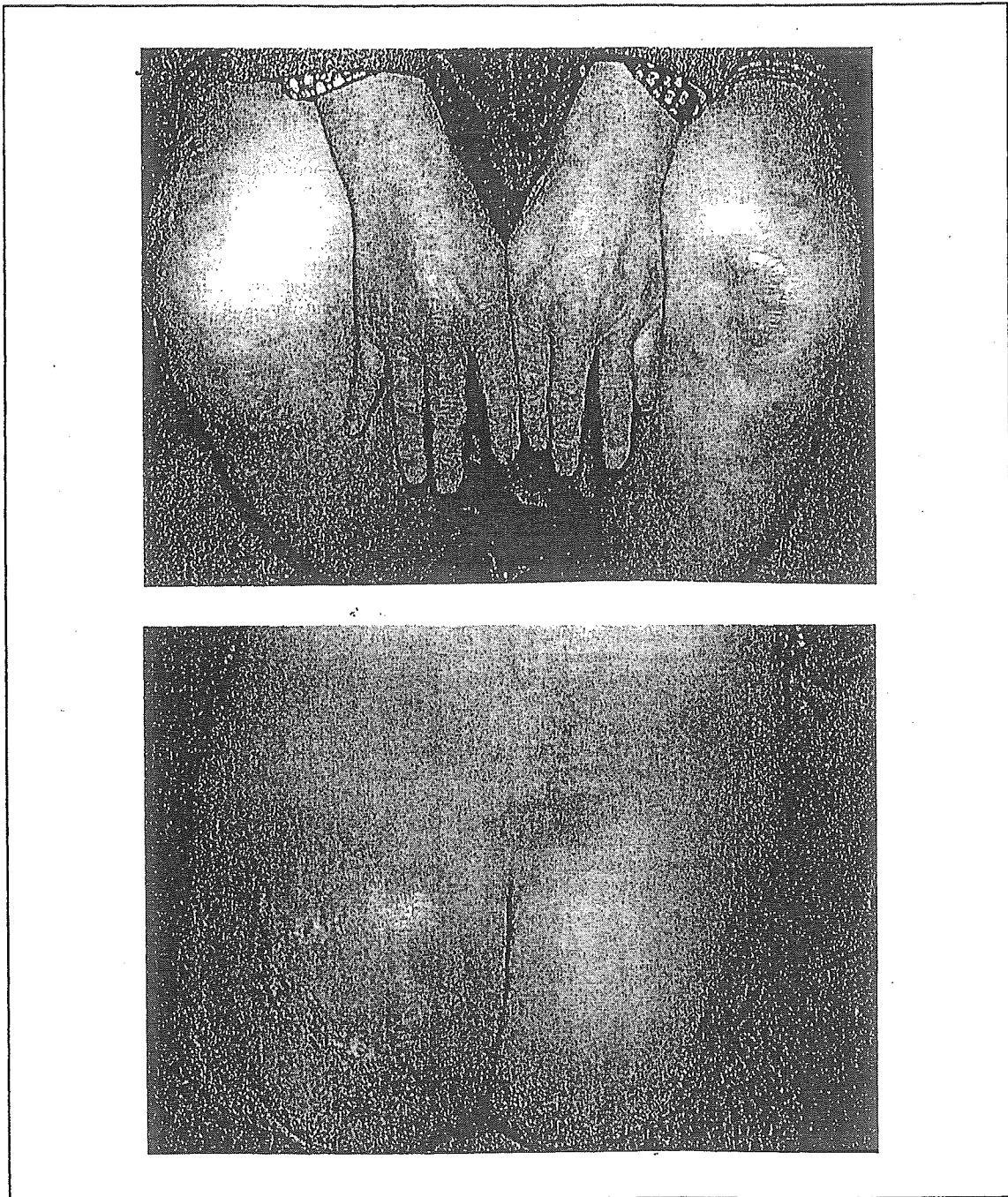
Detection of drug resistance to antileprosy drugs is frequently observed in relapsed cases (3,7), therefore, the relapsed case examined in this study could be predicted to be resistant to some drugs.

Several mutations in the *folP1* gene were

Figure 2. The mutation in *folP* at codon 55 (CCC→TCC). A: Wild type. *M. leprae* Thai- 53 B: *M. leprae* isolate from the relapsed case.



Picture 1-2. Active skin lesions on the knees and gluteal region



reported at codons 53 and 55, namely ACC→GCC, AC→ATC at codon 53 and CCC→CTC, CCC→CGC at codon 55 in high level of dapsone resistant *M. leprae*, which was confirmed by mouse footpad method (6,7,8,12). The mutation detected in this isolate was CCC→TCC at codon 55, substituting proline to serine. This mutation

was recently revealed to associate with the intermediate level of dapsone resistance in clinical samples from a Japanese patient (9). This is the first case of dapsone resistant leprosy case detected by molecular biological analysis in Turkey so far.

Detection of mutations by sequence analysis

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is a simple and rapid method compared with that by mouse footpad test to investigate the susceptibility to antileprosy drugs. We recommend surveying the prevalence of drug resistant *M. leprae* from leprosy patients especially from the relapsed and/or intractable cases for effective treatment and the control of leprosy in Turkey.

#### ACKNOWLEDGMENT

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# ハンセン病の分子疫学

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キーワード：ハンセン病、らい菌、遺伝子型別、感染経路

## はじめに

らい菌およびハンセン病に対する分子生物学の応用は *Mycobacterium leprae* の蛋白質に対するモノクローナル抗体の作成とその遺伝子のクローニングならびに塩基配列の決定に始まった。さらにゲノムDNAの全塩基配列が決定され、それらは Polymerase Chain Reaction (PCR) の確立に応用され、遺伝子多型に基づいた型別法の開発も図られた。これらの手法を用いたらい菌の伝播に関する解析により、流行地域での多数の住民への感染、異なる遺伝子型のらい菌の分布の実態が明らかとなってきた。これらの成果のなかにはハンセン病の感染、とりわけ感染源についてこれまでに考えられてきた概念の見直しを示唆するものもいくつかあり、今後のハンセン病対策上重要な意味をもつことが考えられる。筆者のらい菌の型別に関する分析とともにらい菌の分子生物学の成果のハンセン病の疫学解析への応用と得られた知見について述べる。

## Polymerase Chain Reaction(PCR)の らい菌感染実態解明への応用

*Mycobacterium leprae* (らい菌) ゲノム遺伝子の全塩基配列がColeらによって2001年に決定、公開されたが<sup>1)</sup>、それより以前かららい菌に対するモノクローナル抗体<sup>2)</sup>の作成とそれによって認識される蛋白質の遺伝子のクローニングにより、部分的にらい菌の遺伝子塩基配列が明らかにされてきた<sup>3,4,5)</sup>。それらは当然、すでに報告されていたPCRのらい菌への応用に利用された。545塩基の共通のコアを有するらい菌の繰り返し配列<sup>6)</sup>は最初、65kDa蛋白質の遺伝子の下流部に見出されたが、らい菌ゲノム中に約30個存在することが明らかとなり、それを標的としたWoodsらによるらい菌のPCRが最初に報告されてから<sup>7)</sup>、いくつかのPCRの方法が考案、報告された<sup>8,9,10)</sup>。

らい菌が *in vitro* で培養不可能であることにより、ハンセン病におけるその動態について解析を行う手段が無く、その把握と伝播の解析はそれまで不可能であったが、PCRはその特異性と感度の両面でそのために必要とする条件を満たし、ハンセン病の高い有病率を示す地域での住民の鼻粘膜上でのらい菌の存在の検出、あるいはハンセン病の流行地域と非流行地域でのらい菌の分布の比較に応用され、血清疫学の解析からも推察されていた患者以外でのらい菌の存在を強く示唆する知見を加えた。

流行地域・非流行地域住民における鼻粘膜上の

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らい菌の分布。流行地域における住民のらい菌に対する抗体陽性率から、患者以外の一般住民も広範囲のらい菌の感染を受けていることが考えられてきた<sup>11, 12, 13, 14)</sup>。また鼻粘膜がらい菌の侵入部位として重要であることが指摘されている<sup>15, 16)</sup>。これらの知見と関連してらい菌の感染実態の解明のために鼻粘膜上のらい菌の存在がPCRによって調べられた。

Pattynらは患者および家族内接触者の鼻粘膜について調べ<sup>17)</sup>、少菌型と多菌型との接触者の間にらい菌特異的なPCRの陽性率に差が無く、また治療した多菌型の接触者でも陽性例があったことから感染は患者からではなく、生活する社会環境からの感染が重要であると述べた。

多菌型患者、流行地（フィリピン）において職業的に患者と接触するグループ、流行地での一般住民、非流行地（オランダ）の住民の鼻粘膜からのらい菌DNAの検出率の比較が de Witらによって行われた<sup>18)</sup>。多菌型患者の鼻粘膜からは多数の抗酸菌が排出されることが観察されたが、未治療患者の55%がPCR陽性を示し、また患者鼻汁中には多数のらい菌が存在することが証明された。流行地の患者との接触者と一般住民間のPCRの陽性率に有意差は無く、一方非流行地の住民は全員、陰性を示した。これらの結果から流行地におけるらい菌の広い伝播が示され、流行地での何らかのりザーバでの感染源の維持とそれへの暴露による汚染が新たな感染を起こしていると推察した。

Kaltserらはインドネシアの南スラウェシで住民の鼻粘膜上のらい菌DNAの検出を行った結果、7.8%の住民がPCR陽性を示した<sup>19)</sup>。また患者と同一家屋に居住する接触者と非接触者とのPCRの陽性率には有意差が見られなかった。これらの結果もハンセン病の有病率の高い地域でのらい菌の広い分布と患者以外の感染源の存在を強く示した。

さらに、佐伯らは北マルク地方の有病率が2.8から9.4%のハンセン病の高い流行を示す4集落の患者、住民の合計1,199名について鼻粘膜からのらい菌DNAの検出を行った<sup>20)</sup>。一般住民の26.6%からPCR陽性の結果が得られ、また32.2%の患者（PB15/40, MB4/19）が陽性であった。治療終了者は42.9%（6/14）が陽性であり、これら3群の

間に有意差は示されなかった。この観察では家族内接触者と非家族内接触者の間に陽性率の有意差（ $P=0.02$ ）が見られたが、これは接触の有無よりもむしろ同一家族は同一の感染源に暴露されていることによりこのような結果が認められたと解釈するほうが妥当であると考えられる。多くの解析の結果は何らかのりザーバの存在を強く示唆しているが、今後感染源あるいはりザーバが何であるかを証明することが望まれる。以前よりらい菌が水中に存在するのではないかとわれていた<sup>21)</sup>。またらい菌以外の抗酸菌については水中での存在とそこからの感染が多数報告されている<sup>22, 23, 24)</sup>。Matsuokaらは佐伯による本研究と平行して行った観察で<sup>25)</sup>、井戸水からのらい菌DNAの検出とそれらを生活用水に用いている住民における有意に高い有病率から、流行地域における生活用水の感染源としての重要性を指摘した。

いずれの観察もハンセン病流行地域では患者以外に多くの住民がらい菌を鼻粘膜上に有することを示し、その知見は疫学的観察から得られた推論<sup>26)</sup>、あるいは血清疫学の観察結果から得られた感染と発病に関する考察<sup>11, 12, 13, 14)</sup>、即ちハンセン病流行地域においては多くの住民がらい菌の感染を受けており、その中のごく一部がハンセン病を発症するのであり、またその感染源をハンセン病患者以外に求めることにより、よく観察結果を説明できるとした考えを細菌学的に強く裏付ける結果であった。WHOによる多剤併用療法は患者の治療と同時に多菌型患者を治療することにより感染源が除去され、新たな感染の発生を防ぐことも意図されていた。しかしながらこの対策が世界的規模で導入されて20年を経過しても一向に新患者の減少が見られないことも、感染源を多菌型患者に求めることの不自然さの側面であるように思われる。

## らい菌の遺伝子型別と疫学解析

感染症の疫学解析を行うことにより新たな感染防止策を講ずるためには、その感染源の特定あるいは感染経路の解明を図ることが必須の条件であるが、そのためには個々の感染例からの分離株を型別することが基本的な手法となる。そのために、

多くの病原微生物について様々な型別法が開発され、応用されてきたが、らい菌は遺伝子の多様性が極めて乏しく他の微生物で有用な型別法は応用することができなかつた<sup>27,28)</sup>。近年ゲノムマップ上の2,785kbに位置するTTC配列のコピー数が分離株間で異なることと<sup>29)</sup>、*rpoT* 遺伝子内に多型性が見出され、らい菌は二つの遺伝子型に分類されることが相次いで報告された<sup>30)</sup>。ハンセン病についても、それらの多型性を利用したらい菌分離株の型別と疫学解析がようやく緒についた。

TTC繰り返し数の多様性と疫学解析。Shin<sup>30)</sup>らはらい菌の蛋白質非コード領域にTTCの3塩基からなる繰り返し配列があり、フィリピンのセブにおいて分離されたらい菌がそれぞれ10コから37コの異なる繰り返し数を有することを示した。筆者はインドネシアの北マルクおよび北スラウェシ地方の患者の病変部あるいは住民の鼻粘膜から得たらい菌についてTTC繰り返し数の違いによる型別を行い、ハンセン病の感染について解析を試みた。上記、佐伯の行った解析<sup>20)</sup>に用いられた北マルクの1集落の患者および住民の51例の鼻粘膜上のらい菌は10から14コピーのTTC繰り返しを示した。8軒の住居について同居する住民患者の複数の型別が可能であった。そのうち5軒には元患者、患者、発症を疑われる人が同居していたが、それらの人たちと同一住居に居住する人の鼻粘膜からのらい菌は元患者、患者とは異なるTTC遺伝型を示した。更に興味あることに2組の父親とその息子がハンセン病である例について病変部位から得たらい菌のTTC遺伝子型を比較したところ、1組の親子はいずれも10コピーであったが、他の1組は父親からのらい菌が18コピー、その息子からのらい菌は10コピーのTTC遺伝子型を示した<sup>31)</sup>。

これまでハンセン病は患者とりわけ多菌型患者との濃厚接触が感染の原因だといわれ、疫学観察の結果では多菌型患者との住居内接触は非接触に比し、9倍の危険率であったとする報告もある<sup>32)</sup>。しかしその一方で、北マラウイでの80,000人を対象とした観察で、接触者からの新患数は全体の15%であり、大多数は認められる接触層が無かった人であったという報告がある<sup>33)</sup>。仮に家族内濃厚接触が感染の原因であるとするならば、同一家族、また特に親子同士の感染例からはすべて同じ

遺伝子型のらい菌が分離されるはずである。われわれが得た住民、患者におけるTTC遺伝子型別の結果は家族内濃厚接触による感染が主要感染機会であることを否定するものであり、それよりも鼻粘膜上のらい菌の伝播についてPCRによって調査した結果導き出された、感染源は患者以外の住居外にあり、多くの住民はらい菌に暴露されている結果、らい菌の汚染を受けるとする推察と完全に一致していた。そうであれば、なぜ特定の家族に複数の患者の発生が見られるかが問題となるが、それは例えば Toll-like-receptor<sup>34)</sup> などにより規定される高感受性形質の遺伝的集積の結果、特定の家族集団に症状の発現が多く認められるであろうと説明することが可能である。

以上のようにハンセン病の感染様式に関して、近年有用となった手法を用いた疫学解析の結果はこれまで信じられてきた患者を唯一の感染源とする説とそれに基づく対策の見直しを強くせまっている。今後より明確な解析行われ、抜本的ハンセン病対策が構築されることを期待する。

*rpoT* 遺伝子多型とそれらの地理的分布。Matsuokaらはヌードマウス足跡内での異なる増殖速度に着目し、*rpoT* 遺伝子内の配列に異なる2つの遺伝子型があることを見出した<sup>29)</sup>。即ち、アスパラギン酸、イソロイシンに相当するGACATCの6塩基配列を3個直列する株(3型)と4個直列する株(4型)に分けられた。それに基づいて国内、国外から得たらい菌についてその遺伝子型を観察したところ、日本の本州、韓国、中国東北部には圧倒的に4型が多く分布し(図1)、沖縄および東アジア以外での世界の各地では3型が多数であり(図2)、東アジアにおける4型の優位は極めて特徴的であった。このような民族あるいは地域によって特異的な微生物の遺伝子型が分布することはよく知られており、それは過去の人類の移動あるいは民族の形成と密接に関連して形成されたと考えられている<sup>35,36)</sup>。東アジアの4型の優位な分布は現在の日本人が成立した過去の歴史と密接に関連して形成されたものと考えられた。

現代の日本人の成立した説としては埴原によって提唱された2重構造モデル<sup>37)</sup>が最も広く受け入れられている。それによると日本人は以下の過程