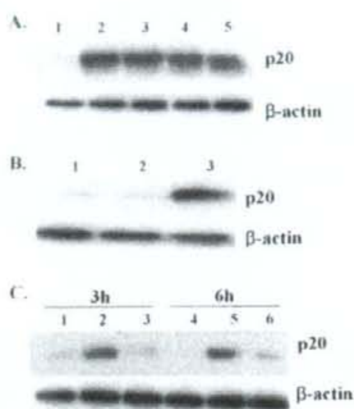

FIG. 9. Activation of caspase-1 by rPLY and *Streptococcus pneumoniae* infection. Peritoneal macrophages were incubated with biotinylated YVAD-cmk (30 μM) for 1 h and subsequently stimulated with rPLY, cholesterol-treated rPLY, or LPS for 3 h, using different concentrations of rPLY or LPS. Activated caspase-1 was precipitated using streptavidin beads. Precipitates were subsequently analyzed for the presence of active caspase-1 (p20 subunit) by Western blotting using a caspase-1 antibody (A and B). (A) Lanes: 1, unstimulated; 2, 1 μg/ml rPLY; 3, 0.1 μg/ml rPLY; 4, 1 μg/ml cholesterol-treated rPLY; 5, 0.1 μg/ml cholesterol-treated rPLY. (B) Lanes: 1, unstimulated; 2, 1 μg/ml LPS; 3, LPS plus ATP (1 mM). (C) Similarly, peritoneal macrophages were incubated with biotinylated YVAD-cmk (30 μM) for 1 h and infected with wt *S. pneumoniae* and the Δ*ply* mutant at an MOI of 10, and cell lysates were collected at the indicated time points. Activated caspase-1 was detected as mentioned above. Lanes 1 and 4, uninfected cells; lanes 2 and 5, wt *S. pneumoniae*-infected cells; lanes 3 and 6, Δ*ply* mutant-infected cells. For all panels, results are representative of at least three separate experiments.

rule out the contamination of our rPLY preparations with ATP, the level of ATP was determined using the Enliten ATP assay system (Promega). The ATP level in 1 μg/ml of rPLY was <17.9 pM, and this concentration of ATP never enhanced caspase-1 activation in LPS-stimulated macrophages (data not shown). These results suggest that rPLY induces the activation of caspase-1 without any other additional stimuli and that this ability is independent of its cytolytic activity (Fig. 9A). When macrophages were infected with wt *S. pneumoniae*, caspase-1 activation was strongly induced, whereas the Δ*ply* mutant was unable to induce it effectively (Fig. 9C), suggesting that PLY is also required for the activation of caspase-1 in macrophages infected with *S. pneumoniae*.

DISCUSSION

In the present study, we examined the role of PLY in the induction of various proinflammatory cytokines in response to *S. pneumoniae* infection. Among a number of different cytokines induced in macrophages infected with *S. pneumoniae*, the production of IL-1α, IL-1β, and IL-18 was exclusively dependent on the presence of the *ply* gene, as clearly shown using an in-frame deletion mutant of *ply*. In contrast, deletion of *ply* did not result in any severe change in the ability of *S. pneumoniae* to induce the production of TNF-α, IL-6, and IL-12. This finding strongly implied that the PLY protein itself is an effective ligand that activates caspase-1, which is required for the maturation of IL-1β and IL-18. This possibility was confirmed by the fact that the rPLY protein alone did induce the production of these cytokines. The reduced production of IL-1α, IL-1β, and IL-18 observed in macrophages infected with the Δ*ply* mutant recovered when it was supplemented with rPLY, to a level comparable with that in macrophages infected with wt *S. pneumoniae*. Taken together, these findings clearly show that the profile of the *S. pneumoniae*-induced cytokine response strongly depends on PLY and that PLY is unique in terms of activity for inducing caspase-1 activation, which is not observed with LPS.

Various bacterial ligands are recognized by several TLRs, with a strict specificity of each TLR and the pattern of each ligand. TLR4, which plays an essential role in LPS recognition, is also reported to be involved in the recognition of PLY (27, 51) and other related bacterial cytolysins, including anthrolysin (41) and *Listeria*-derived cytolysins (20). In the present study, using rPLY, we demonstrated that TLR4 was essential for the PLY-induced production of IL-1α, IL-1β, and IL-18. Interestingly, in spite of their similar and exclusive dependence on TLR4, rPLY and LPS induced quite different profiles of cytokine production. This suggested that PLY and LPS differentially activated the downstream signaling cascades. Indeed, there was some difference in the timing and duration of NF-κB activation between macrophages stimulated with rPLY and LPS. Furthermore, PLY only weakly activated ERK and p38 MAPK, whereas LPS strongly activated these downstream molecules. Because both NF-κB and MAPKs contribute to the induction of various cytokines, the different modes of activation of these signaling molecules might account for the difference observed in cytokine-inducing activity between PLY and LPS. For example, the production of TNF-α is regulated by NF-κB and MAPKs during and after transcription. ERK is involved particularly in the posttranscriptional transport of TNF-α mRNA from the nucleus to the cytoplasm in response to LPS (15). Although the exact reason that rPLY induced just a small amount of TNF-α is not yet clear, it is possible that TNF-α production was down-regulated posttranscriptionally, because rPLY clearly induced the gene expression of TNF-α but hardly activated ERK.

Although both LPS and PLY are TLR4 agonists, the precise interaction of each ligand with TLR4 seems to be different. Malley et al. (27) showed that MD2, which associates with TLR4 and plays a critical role in the cytokine response to LPS, was not required for PLY-dependent activation of NF-κB. Furthermore, Srivastava et al. (51) demonstrated that there is a specific and strong physical interaction between TLR4 and PLY by using a solid-phase binding assay. It is likely that cofactors required for LPS sensing via TLR4, such as MD2, LPS-binding protein, and CD14, are not so important for the recognition of PLY by TLR4. Sa15-21, which is an anti-TLR4 monoclonal antibody, was reported to bind to the N-terminal leucine-rich repeat of TLR4 and to act as an agonistic antibody. This antibody strongly induced NF-κB activation but modestly induced TNF-α production in vivo and in vitro (2). Therefore, it is conceivable that the observed difference in the activation of the downstream signaling cascade and the overall difference in the profile of cytokine production may result from the different interaction between TLR4 and LPS or PLY.

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LPS directly binds to MD2 associated with the extracellular domain of TLR4 and then induces clustering of TLR4/MD2, which leads to the activation of two main signaling pathways downstream of TLR4, namely, the MyD88/Toll–IL-1 receptor (TIR) domain-containing adaptor protein (TIRAP)-dependent pathway and the TIR domain-containing adaptor inducing beta interferon (IFN-β) (TRIF)/TRIF-related adaptor molecule (TRAM)-dependent pathway (46). The MyD88/TIRAP-dependent pathway is involved preferentially in the induction of proinflammatory cytokines, whereas the TRIF/TRAM-dependent pathway is involved in the induction of IFN-β rather than proinflammatory cytokines (3). Although it remains unclear whether TLR4/MD2 clustering occurs in response to PLY, our data suggested that MyD88 was required for the rPLY-induced expression of proinflammatory cytokines. A finding that rPLY did not induce the expression of IFN-β (data not shown) implied that PLY may activate the MyD88/TIRAP-dependent but not the TRIF/TRAM-dependent pathway via its binding to TLR4. It has been reported that the integrity of lipid rafts is essential for the cellular response to LPS and that TLR4 is recruited to lipid rafts after stimulation with LPS (52). Do TLR4/MD2 clustering and/or recruitment of TLR4 to lipid rafts occur upon stimulation with PLY? Are lipid rafts required for the TLR4-dependent cytokine response to PLY? Which part of TLR4 interacts with PLY, and does binding result in some conformational change of TLR4 which leads to activation of the downstream signals? Although there are a number of questions to be addressed, a PLY-induced, noncanonical pathway may provide a new insight into the study of TLR signaling, especially that via TLR4.

An interesting finding of this study is that the caspase-1-dependent cytokines, IL-1β and IL-18 were strongly induced by stimulation with rPLY but hardly induced by LPS alone. Our study confirmed that PLY-induced production of IL-1β and IL-18 was caspase-1 dependent, and the active form of caspase-1 was detected in macrophages stimulated with rPLY. The question of whether TLR4 is involved in the activation of caspase-1 arose. To clarify this point, we tested the involvement of TLR4 in PLY-induced caspase-1 activation. When macrophages were stimulated with rPLY at a sublytic concentration (0.1 μg/ml), our result suggested a requirement for TLR4 in caspase-1 activation, but TLR4 dependency was not observed when cells were stimulated with rPLY at a lytic concentration (1 μg/ml) (data not shown). Our assumption is that both TLR4-dependent and -independent pathways might be involved in PLY-induced activation of caspase-1 and that TLR4 might potentiate the activation of caspase-1. The decrease in intracellular K^+ level induced by danger signals and toxins, such as ATP and nigericin, results in the enhancement of caspase-1 activation (44). Furthermore, a cytolytic concentration of PLY caused a K^+ efflux without inhibiting the activity of Na^+,K^+-ATPase (11). It is therefore possible that a PLY-formed pore may mediate K^+ efflux and consequently modulate the caspase-1 processing pathway. However, the ability of rPLY to induce the production of IL-1β and IL-18 was not affected, even when its pore-forming activity was blocked by cholesterol pretreatment. Moreover, rPLY-induced activation of caspase-1 was not abolished by cholesterol pretreatment. Because cholesterol pretreatment completely blocked the cytolytic activity of 0.1 μg/ml rPLY and this concentration of cholesterol-treated rPLY clearly induced caspase-1 activation and the production of caspase-1-dependent cytokines, pore formation does not appear to be essential for the induction of caspase-1 activation by PLY. Nonetheless, we cannot completely rule out the possibilities that pore-forming activity of rPLY might remain at a level under the detection limit of the lactate dehydrogenase release assay, even after cholesterol treatment, and that the level of pore formation might be sufficient to mediate K^+ efflux that can induce caspase-1 activation. In a future study, we plan to clarify whether the PLY-formed pore is essentially involved in the activation of caspase-1 by using a truncated rPLY that completely lacks cytolytic activity. Indeed, a noncytolytic mutant of PLY has been reported to retain its ability to activate TLR4 signaling and to induce IL-6, TNF-α, and IFN-γ (5, 27), and therefore such a mutant protein would be useful for this line of investigation. PLY is a multifunctional protein and its mechanisms for cytolysis have been investigated, but how this cytolysin induces caspase-1 activation remains to be clarified.

Both IL-1 and IL-18 have been reported to play a key role in infections caused by *S. pneumoniae*. Zwijnenburg et al. (55) demonstrated that endogenous IL-1 is essential for an adequate host defense in pneumococcal meningitis, as reflected by impaired bacterial clearance and reduced survival of IL-1 receptor-deficient (IL-1R$^{-/-}$) mice. Similarly, in a murine pneumococcal pneumonia model, IL-1R$^{-/-}$ mice showed an impaired early host defense (45). Studies have also shown that IL-18 has a protective role in the early immune response in a murine pneumococcal pneumonia model by promoting bacterial clearance from the lung and delaying the progression to a systemic infection (26), although the contribution of an inflammatory response to the detrimental effect was also reported for a meningitis model (56). In the present study, PLY was identified as an essential factor that contributes to IL-1α, IL-1β, and IL-18 production in response to *S. pneumoniae*.

It is therefore possible that PLY-dependent caspase-1 activation and subsequent production of IL-1β and IL-18 protect the host against pneumococcal infection. The present finding that IL-18 production induced by *S. pneumoniae* was dependent on TLR4 may account for the increased susceptibility to *S. pneumoniae* of mice that lack functional TLR4 (27). A previous report demonstrated that the interaction between PLY and TLR4 resulted in the induction of caspase-dependent host cell apoptosis in mice infected with *S. pneumoniae* (51), although several reports demonstrated that apoptosis-like cell death induced by *S. pneumoniae* or PLY in various types of cells is independent of caspases (6, 10, 12). In that report (51), it appeared that *S. pneumoniae*-induced apoptosis contributed to the host defense, because administration of the broad-spectrum caspase inhibitor z-VAD-fmk to mice increased the mortality rate after pneumococcal colonization in the nasopharynx. Because PLY-induced IL-18 production was also inhibited by z-VAD-fmk in our study, IL-18 production in addition to apoptosis may participate in the host defense mechanism induced by the TLR4-PLY interaction.

IL-1 and IL-18 are proinflammatory cytokines that can induce inflammatory reactions and are involved in the development of systemic and local inflammatory illnesses, including arthritis, asthma, sepsis, pneumonia, and meningitis caused by various agents (13, 30, 38, 42, 56). From our finding that PLY,

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which is a toxin highly associated with the pathogenicity of *S. pneumoniae*, plays an essential role in the activation of caspase-1 and subsequent induction of IL-1 and IL-18 production upon *S. pneumoniae* infection, it is thus suggested that the PLY-dependent cytokine response comprises part of the pathophysiological mechanism, in addition to cytolytic activity and other functions of the cytolysin as a virulence factor. Indeed, the absence of IL-1R or IL-18 affected the histopathology of the lungs and brains in murine models of *S. pneumoniae* infection (26, 55). Analysis of infection models using mice deficient for caspase-1 or both IL-1R and IL-18 would elucidate the significance of PLY-dependent cytokines in the aspects of host defense and disease progression. For better control of the diseases caused by *S. pneumoniae* infection, more detailed investigations into the virulence of this pathogen, host defense mechanisms, and their influences on pathophysiology are required. A full understanding of the functions and physiological roles of PLY may pave the way for the control of harmful cytokines during pneumococcal infection.

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Dependency of Caspase-1 Activation Induced in Macrophages by *Listeria monocytogenes* on Cytolysin, Listeriolysin O, after Evasion from Phagosome into the Cytoplasm[1]

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Listeriolysin O (LLO), an *hly*-encoded cytolysin from *Listeria monocytogenes*, plays an essential role in the entry of this pathogen into the macrophage cytoplasm and is also a key factor in inducing the production of IFN-γ during the innate immune stage of infection. In this study, we examined the involvement of LLO in macrophage production of the IFN-γ-inducing cytokines IL-12 and IL-18. Significant levels of IL-12 and IL-18 were produced by macrophages upon infection with wild-type *L. monocytogenes*, whereas an LLO-deficient mutant (the *L. monocytogenes* Δ*hly*) lacked the ability to induce IL-18 production. Complementation of Δ*hly* with *hly* completely restored the ability. However, when Δ*hly* was complemented with *ilo* encoding ivanolysin O (ILO), a cytolysin highly homologous with LLO, such a restoration was not observed, although ILO-expressing *L. monocytogenes* invaded and multiplied in the macrophage cytoplasm similarly as LLO-expressing *L. monocytogenes*. Induction of IL-18 was diminished when pretreated with a caspase-1 inhibitor or in macrophages from caspase-1-deficient mice, suggesting the activation of caspase-1 as a key event resulting in IL-18 production. Activation of caspase-1 was induced in macrophages infected with LLO-expressing *L. monocytogenes* but not in those with Δ*hly*. A complete restoration of such an activity could not be observed even after complementation with the ILO gene. These results show that the LLO molecule is involved in the activation of caspase-1, which is essential for IL-18 production in infected macrophages, and suggest that some sequence unique to LLO is indispensable for some signaling event resulting in the caspase-1 activation induced by *L. monocytogenes*. *The Journal of Immunology,* 2008, 180: 7859–7868.

Listeria monocytogenes (LM)[3] is a Gram-positive facultative intracellular bacterium that often causes life-threatening infections in immunocompromised hosts, including newborns and elderly people (1–4). The pathogenicity of LM can be attributed to the invasion and subsequent intracellular parasitism in a variety of host cells such as hepatocytes, fibroblasts, and epithelial cells. Professional phagocytes, such as macrophages, are also the major target cells of LM because the pathogen can survive and grow inside macrophages, even once being trapped in phagosomes after phagocytosis. Virulence factors encoded in *Listeria* pathogenicity island 1 are required for the evasion of intracellular bactericidal mechanisms by LM. Among the group of virulence factors, listeriolysin O (LLO), a 56-kDa cytolysin encoded by *hly*, is the most important virulence determinant and plays an essential role in bacterial escape from the phagosome into the cytoplasm where the pathogen multiplies efficiently (5–7).

In mice infected with LM, innate immune cells such as macrophages and dendritic cells are activated to release proinflammatory cytokines, including TNF-α, IL-1, and IL-6. In addition to these proinflammatory cytokines, IL-12 and IL-18, which are IFN-γ-inducing cytokines, are also released from innate immune cells and subsequently induce the production of IFN-γ from NK cells and NK dendritic cells (8, 9). Such an initial IFN-γ response is not only essential for the host defense against primary LM infection but is also important for the establishment of T cell-mediated acquired immunity, which is required for the protection of the host against secondary challenge with LM (10, 11).

In contrast to the established role of IFN-γ for the host defense, the mechanism of IFN-γ induction in the initial stage of infection with LM has been elucidated only partially. On the basis of the fact that an infection with the LM strain lacking LLO, which is incapable of escape into the cytosol and intracellular multiplication, never induces a significant level of IFN-γ response (12, 13), LLO seems to play a critical role in the induction of IFN-γ. Regarding the contribution of LLO to the induction of IFN-γ response, there may be several possibilities. One possibility is that LLO serves just as cytolytic protein and simply enables the bacteria to escape from the phagosomal compartment. Then the recognition of the bacterial ligand(s) by some cytoplasmic pattern recognition receptor in the macrophage cytoplasm, for example, the Nod-like receptor (NLR), may result in the activation of the signaling pathway required for the induction of IFN-γ-inducing cytokines. Another possibility is the direct stimulation of the signaling cascade by LLO itself as an essential ligand after serving as the protein toxin necessary for

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[3] Abbreviations used in this paper: LM, *Listeria monocytogenes*; ILO, ivanolysin O; LI, *Listeria ivanovii*; LDH, lactate dehydrogenase; LLO, listeriolysin O; MOI, multiplicity of infection; NLR, Nod-like receptor; PEC, peritoneal exudate cell; PEST, Pro-Glu-Ser-Thr; z-YVAD-fmk, *N*-benzyloxycarbonyl-Tyr-Val-Ala-Asp-fluoromethyl ketone.

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evasion into the cytoplasm. As LLO is known to modulate various cellular responses (14), it is likely that the LLO molecule itself may induce or enhance the production of IFN-γ by activating macrophages as a bacterial modulin.

Listeria ivanovii (LI) is an animal pathogen and carries a gene cluster that is highly analogous to the *Listeria* pathogenicity island 1 of LM (15). LI produces ivanolysin O (ILO) encoded by *ilo*, a cytolysin that shows ~80% homology with LLO in amino acid sequence (16). Although LI is capable of evasion into and multiplication inside the macrophage cytoplasm like LM, IFN-γ responses after infection with LI in vitro and in vivo were very low as compared with those induced by LM (17). It is therefore unlikely that only the bacterial entry into the macrophage cytoplasm is sufficient for the induction of IFN-γ production. The comparison between LM and LI may not be the best tool to test the second possibility mentioned above as these two species are not isogenic although they belong to the same genus *Listeria*, and there may be some critical difference in the ligands other than the difference between LLO and ILO. To overcome this problem, isogenic LM mutants producing LLO or ILO were constructed by gene complementation of an LLO-deficient LM mutant with *hly* and *ilo*. In our previous study using these isogenic mutants, we examined whether the initial IFN-γ response is due simply to the entry of LM into the macrophage cytoplasm or whether the presence of LLO itself is required. It was found that LLO-producing LM, but not ILO-producing LM, strongly induced the production of IFN-γ on LM infection in vitro and in vivo (11). The results clearly indicated that the LLO molecule is involved by itself in the induction of host IFN-γ response and not by enabling bacterial cells to be delivered into the macrophage cytoplasm.

IFN-γ production in the host as an innate immune response to LM is highly dependent on the release of two major IFN-γ-inducing cytokines, IL-12 and IL-18 (18). In contrast to IL-12 production, which never requires further processing, the release of IL-18 as an active form definitely requires a cleavage of pro-IL-18 by caspase-1 (19). Recent reports have shown that caspase-1 activation is induced efficiently only by LM, which is capable of escaping from the phagosome, but not by the LLO mutant incapable of evasion into the cytosol (20, 21). In a study that emphasized the importance of intracellular parasitism for caspase-1 activation, the cytosolic flagellin of *Salmonella* appeared to be responsible for caspase-1 activation, but no particular component of *Listeria* was identified (22). These findings raised a possibility that LLO itself is one of the candidates for the bacterial molecule responsible for caspase-1 activation.

In this study, we have examined the molecular basis for the induction of IL-12 and IL-18 in macrophages stimulated with LM by using isogenic mutants that differ only in the cytolytic protein, with special reference to the induction of cytokine gene expression and caspase-1 activation.

Materials and Methods

Mice

Female mice of C57BL/6 (normal, TLR4 knockout) strains were purchased from Japan SLC. Caspase-1 knockout C57BL/6 mice were provided by H. Tsutsui (Hyogo Medical College, Hyogo, Japan). Mice were maintained in specific-pathogen-free conditions and used at 7–9 wk of age. All the experimental procedures performed on mice were approved by the Animal Ethics and Research Committee of Kyoto University Graduate School of Medicine, Kyoto, Japan.

Bacterial strains and growth conditions

The parental wild-type LM strain used in this study was *L. monocytogenes* EGD (serovar 1/2a). Three isogenic mutants, Δ*hly*, Δ*hly*::*hly*, and Δ*hly*::*ilo*, were constructed from wild-type LM using the homologous recombination method and the similarity in the expression level of each cytolysin was shown in a previous study (11). Bacteria were grown overnight in brain-heart infusion broth (EIKEN Chemical) at 37°C with shaking. One volume of the overnight culture was added to 100 volumes of fresh brain-heart infusion medium and cultured further for 5 h. Bacterial cells were washed, suspended in PBS supplemented with 10% glycerol, and stored in aliquots at −80°C. The concentration of bacteria was determined by plating 10-fold serially diluted suspensions on a tryptic soy agar (EIKEN Chemical) plate and counting the number of colonies after cultivation for 24 h.

Cells

Peritoneal exudate cells (PECs) of mice were obtained 3 days after an i.p. injection of 2 ml of thioglycolate medium (EIKEN Chemical). After washing with RPMI 1640, PECs were incubated on culture plates at 37°C for 3 h in culture medium that consisted of RPMI 1640 supplemented with 10% FCS. After incubation, the cells were washed with RPMI 1640 and adherent PECs were used for infection study. Bone marrow cells were obtained from tibiae of mice and then cultured in RPMI 1640 supplemented with 10% FCS, gentamicin (10 μg/ml; Wako Pure Chemical Industries), and recombinant mouse M-CSF (100 ng/ml; R&D Systems) for 5 days. After washing with RPMI 1640, adherent bone marrow-derived macrophages were collected. The cells were plated at 1.5×10^5 cells/well in 96-well microplates or at 2×10^6 cells/well in 6-well microplates for detection of cytokines or active caspase-1, respectively. The cells were infected with bacteria at a multiplicity of infection (MOI) of 1 for 30 min at 37°C.

Immunofluorescence analysis of bacterial escape

Adherent PECs were seeded into a 24-well plate at 5×10^5 cells/well and then infected with bacteria at a MOI of 1 for 30 min at 37°C. Cells were washed three times and cultured for 3 h at 37°C in the presence of 10 μg/ml gentamicin. After several washings, the cells were fixed by 3% paraformaldehyde and incubated overnight at 4°C with a blocking solution that is PBS containing 10% Blocking One (Nacalai) and 0.1% saponin (Nacalai). F-actin formation was visualized by the staining of infected cells with Alexa Fluor 488-phalloidin (Invitrogen), and the bacterial cell was stained by treatment with rabbit anti-*Listeria* polyclonal Ab (ViroStat) in blocking solution at room temperature for 1 h in a dark room and then with Alexa Fluor 594-anti-rabbit IgG Ab (Invitrogen) at room temperature for 1 h in a dark room. Cells were examined under fluorescent microscope for actin cloud or actin tail formation.

Neutralization of cytokines

Neutralization of cytokines in culture was done as reported previously (23, 24). The neutralizing Ab specific for IL-12 (goat, polyclonal) and that for IL-18 (rat, clone 93-10C) were purchased from R&D Systems and Medical & Biological Laboratories, respectively. As control Abs, normal rat IgG (ICN Biomedicals) and normal goat IgG (R&D Systems) were used. After infection with bacteria for 0.5 h, Abs were added to cell culture medium at 5 μg/ml.

ELISA

Levels of cytokines in culture supernatants were determined by two-site sandwich ELISA as reported previously (11, 25). Briefly, bacteria were added to the cell cultures and incubated at 37°C for 30 min. The infected cells were cultured for an additional 24 h in the presence of 10 μg/ml gentamicin. Culture supernatants were then collected and stored at −80°C until the cytokine measurement. The ELISA kit for TNF-α was purchased from eBiosciences. For the titration of other cytokines, pairs of biotin-labeled and unlabeled mAbs specific to IL-18 (Medical & Biological Laboratories), IL-12p70 (Endogen), and IFN-γ (Endogen) were used.

Quantitative real-time RT-PCR

Total cellular RNA was extracted using NucleoSpin RNA II (Macherey-Nagel), according to the manufacturer's instructions. The collected RNA (0.2 μg) was treated with RNase-free DNase (Promega) to eliminate contaminating DNA before being subjected to reverse transcription using random primers (Invitrogen) and ReverTra Ace (TOYOBO). Quantitative real-time RT-PCR was performed on ABI PRISM 7000 (Applied Biosystems) using Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen). Results were analyzed with ABI PRISM 7000 SDS software. The following mouse primer sequences were designed using Applied Biosystems Primer Express software: *tnfα*, 5′-ATGCTGGGACAGTGACCTGG-3′ (forward) and 5′-CCTTGATGGTGGTGCATGAG-3′ (reverse); *il-12p40*, 5′-GGATGGAAGAGTCCCCCAAA-3′ (forward) and 5′-CTGGAAAAAGCCAACCAAGC-3′ (reverse); *il-18*, 5′-GAAAGCCGCCTCAAACCTTC-3′ (forward) and 5′-CATTGTTCCTGGGCCAAGAG-3′ (reverse);

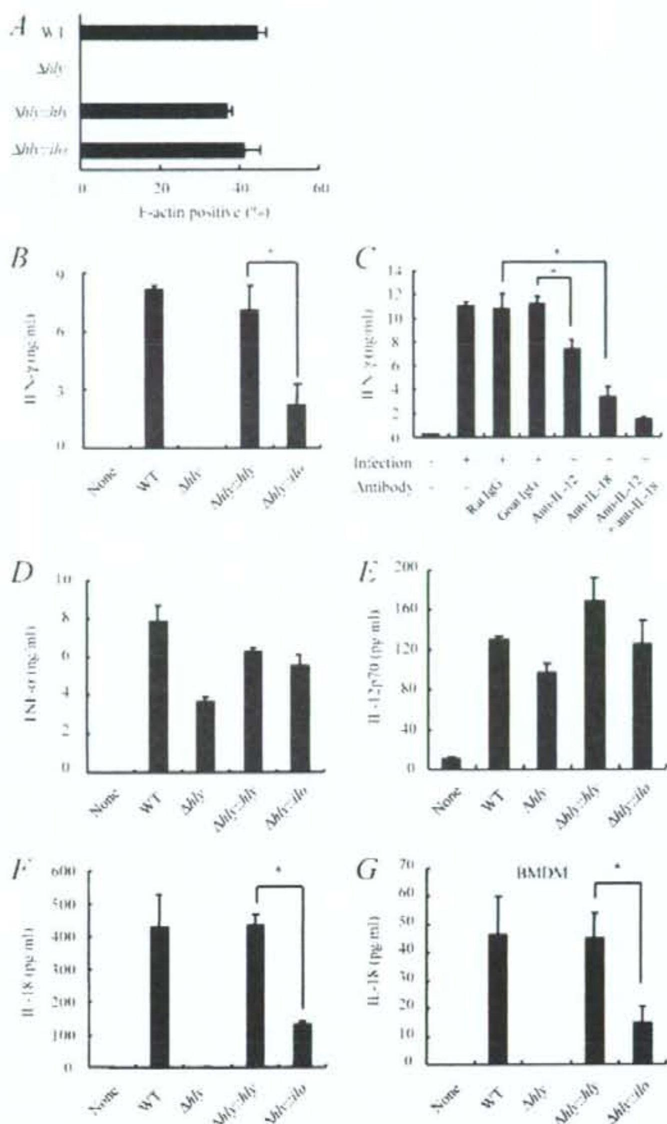

FIGURE 1. LLO- and ILO-expressing LM similarly escape from phagosomes but differently induce IFN-γ and IL-18. Whole PECs (*B* and *C*), adherent PECs (*A* and *D*–*F*), or bone marrow-derived macrophages (*G*) were infected with each LM strain. *A*, Cells were cultured for an additional 3 h in the presence of gentamicin, then bacteria and F-actin were stained and 300 bacteria were counted. The percentage of bacteria positive for associating F-actin was calculated for each strain. The filled bars represent the mean of three independent wells and the error bars indicate the SD. *B*–*G*, Cells were cultured for an additional 24 h in the presence (*C*) or absence (*B*, *D*–*G*) of each neutralizing Ab, and the amounts of each cytokine were then determined by ELISA. Data represent the mean of triplicate assays and SD. Similar results were obtained in three independent experiments. *, $p < 0.01$. WT, Wild type.

caspase-1, 5′-GCCCACTGCTGATAGGGTGA-3′ (forward) and 5′-CCCGGGAAGAGGTAGAAACG-3′ (reverse); and *β-actin*, 5′-GCCCTGAGGCTCTTTTCCAG-3′ (forward) and 5′-TGCCACAGGATTCCATACCC-3′ (reverse). Gene-specific transcript levels were normalized to the amount of β-actin mRNA.

Inhibition of caspase-1

A caspase-1-specific inhibitor, *N*-benzyloxycarbonyl-Tyr-Val-Ala-Asp-fluoromethyl ketone (z-YVAD-fmk), was purchased from R&D Systems. After infection with bacteria for 30 min, this inhibitor dissolved in DMSO was added to cell culture medium at several concentrations. For the control wells, DMSO without inhibitor was added.

Detection of caspase-1

The cells were cultured for several hours at 37°C with gentamicin after infection, with each LM strain at a MOI of 1 for 30 min. After cultivation, supernatants were collected, and cells were lysed in the buffer containing 1% Nonidet P-40 supplemented with 1 μg/ml leupeptin, 1 μg/ml pepstatin A, 1.5 μg/ml aprotinin, and 2 mM DTT. Six milliliters of culture supernatants were precipitated with 7 μg of rabbit polyclonal Ab for mouse caspase-1 p10 (Santa Cruz Biotechnology) in the presence of protein G-Sepharose (GE Healthcare). The cell lysates and precipitates were subjected to SDS-PAGE and subsequently transferred to polyvinylidene difluoride membranes by electroblotting. The membranes were immunoblotted with anti-caspase-1 Ab or anti-β-actin Ab (Sigma-Aldrich).

Detection of lactate dehydrogenase (LDH) release

Culture supernatants were collected, centrifuged, and transferred to new tubes. LDH activity was measured using an LDH cytotoxicity detection kit (TaKaRa BIO). The percentage of LDH release was calculated by using the following formula: percentage of release = 100 × (experimental LDH release − spontaneous LDH release)/(maximal LDH release − spontaneous LDH release). To determine the maximal LDH release, cells were treated with 1% Triton X-100.

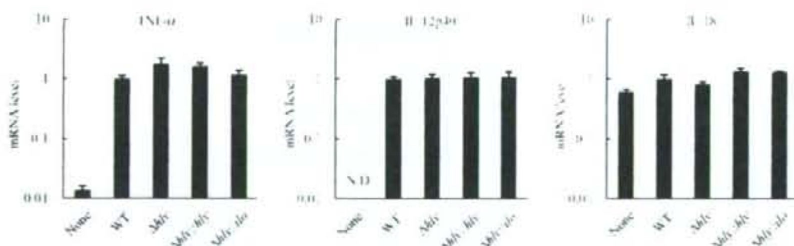

FIGURE 2. Expression of mRNA for various cytokines after infection with LM strains. Adherent PECs were infected with each LM strain. The cells were cultured for an additional 5 h in the presence of gentamicin. Total RNA was extracted and subjected to quantitative real-time RT-PCR for detection of mRNA for TNF-α, IL-12p40, and IL-18. Data represent the mean of triplicate assays and SD. N. D., Not detected; WT, wild type.

Immunofluorescence analysis

Adherent PECs seeded in 24-well microplates at 5×10^5 cells/well were infected with bacteria at a MOI of 1. After cultivation for 21 h, the cells were washed three times with PBS and then fixed in 4% paraformaldehyde. Fragmented DNA was labeled by the TUNEL method using MEBSTAIN Apoptosis Kit Direct (Medical & Biological Laboratories) according to the manufacturer's instructions. Total nucleus was visualized by 4′,6-diamidino-2-phenylindole staining (Dojindo). Bacterial cells were stained by using rabbit anti-*Listeria* polyclonal Ab (VivoStat) and Alexa Fluor 594-labeled anti-rabbit Ig G Ab (Invitrogen). The cells were examined under a fluorescent microscope, and cells positive for each fluorescence were enumerated.

Construction of Δhly::ilo strains expressing full length, truncated, or mutant LLO

An expression vector was constructed by ligation of the *prfA* and *hly* promoter region into the multiple cloning sites of pAT28, which contains a spectinomycin resistance gene, with Ligation High (Toyobo), and then gene fragments of *hly* full length, *hly* $Trp^{492} \rightarrow Ala^{492}$ (W492A), *hly* domain 1–3, and domain 4 were ligated downstream of *hly* promoter. The following primer sequences were used: *prfA*, 5′-cGATGAGCTCTTAATTTAATTTTCCCAAGTAGCAG-3′ (forward) and 5′-ACGCCCCGGGATGAACGCTCAAGCAGAAG-3′ (reverse); *hly* promoter, 5′-CGATCCCGGGAATGGCCCCCTCCTTTGAT-3′ (forward) and 5′-CGCGGTACCGATATCCTTTGCTTCAGTTTG-3′ (reverse); *hly*, 5′-CGATTGCGCATCTGCATTCAATAAAG-3′ (forward); *hly d4*, 5′-CGATCCCGGGAAAATTAACATCGATCACTC-3′ (forward); *hly W492A*, 5′-TTGGGAATGGGCGAGAACGGTAA-3′ (forward); *hly*, 5′-GCTCTAGATTATTCGATTGGATTATCTAC-3′ (reverse); *hly d1–3*, 5′-CGTCTAGATTATGTATAAGCTTTTGAAG-3′ (reverse). The primers for *prfA* were designed to generate restriction sites for *Sac*I and *Sma*I. The primers for *hly* promoter were designed to generate restriction sites for *Sma*I and *Kpn*I/*Eco*RV. The F-primers for *hly* and *hly d4* were designed to generate restriction sites for *Fsp*I and *Sma*I, respectively. The reverse primers for *hly* and *hly d1–3* were designed to generate restriction sites for *Xba*I. The resulting plasmid was introduced into the competent cells of Δ*hly*::*ilo* by electroporation. Transformants were selected on brain-heart infusion agar plates supplemented with spectinomycin (250 μg/ml; Nacalai Tesque). The expression of each LLO molecule produced by transformed Δ*hly*::*ilo* strains was confirmed by Western blotting using anti-LLO Ab, although the Ab showed a weak level of cross-reactivity to ILO. The similarity in the levels of ILO production by these ILO-producing strains carrying the plasmid harboring the *hly* fragment was confirmed by Western blotting using anti-ILO Ab. These polyclonal Abs for LLO or ILO were prepared by hyperimmunization of a normal white rabbit with recombinant LLO or recombinant ILO emulsified in Freund's complete adjuvant.

Statistical analysis

For comparisons between two groups, Student's t test was used when the variances of the groups were judged to be equal by F test. Multigroup comparisons of mean values were made according to the ANOVA and the Fisher's protected least significant difference post hoc test after the confirmation of homogeneity of variances among the groups by using Bartlett's test. Statistical significance was determined as $p < 0.05$.

Results

IL-12- and IL-18-dependent IFN-γ production induced by LM expressing LLO

In the in vitro infection of macrophages prepared from C57BL/6 mice, two Δ*hly* strains complemented with *hly* or *ilo* exhibited a similar level of ability to escape from the phagosome of macrophages (Fig. 1*A*). An essential requirement for LLO in the induction of IFN-γ by LM was confirmed by using whole PECs from C57BL/6 mice, the cell population containing IFN-γ-producing cells such as NK cells (Fig. 1*B*). The IFN-γ-inducing ability of wild-type LM that was abolished by the deletion of *hly* could be restored when Δ*hly* was complemented with *hly* but not *ilo*. These findings were consistent with our previous report in which PECs from C3H/HeN mice were used (11). In the culture system used in this study, both IL-12 and IL-18 were shown to be important for IFN-γ production induced by LLO-expressing LM, as neutralizing Abs against these two cytokines significantly inhibited the IFN-γ response (Fig. 1*C*).

Gene expression and production of IL-12 and IL-18 in response to LM strains

For the assessment of IL-12 and IL-18 production in response to each LM strain, we used culture supernatants of adherent peritoneal macrophages to rule out the possible influences of the products from other nonadherent cells like IFN-γ. A similar level of production of IL-12p70 was observed in all groups of macrophages infected with any of the LM strains used, indicating that induction of IL-12 is not dependent on the escape of bacteria from phagosome and a sort of cytolysin. Another caspase-independent cytokine, TNF-α, was also produced even by macrophages stimulated with Δ*hly* and incapable of evasion into the cytosol (Fig. 1, *D* and *E*). By contrast, the pattern of IL-18 induction by LM strains was quite similar to that of IFN-γ (11). The IL-18-inducing ability of wild-type LM was lost completely by deletion of *hly* (Δ*hly*) but was successfully restored by complementation with *hly* (Δ*hly*::*hly*). Interestingly, such a significant level of restoration was not observed when Δ*hly* was complemented with *ilo* (Δ*hly*::*ilo*) (Fig. 1*F*). The critical difference in the ability to induce IL-18 production between Δ*hly*::*hly* and Δ*hly*::*ilo* was observed also in bone marrow-derived macrophages (Fig. 1*G*). These data indicate that LLO plays an important role in the induction of IL-18 in infection with LM and that the IL-18 response is dependent not on extraphagosomal evasion mediated by either LLO or ILO, but on the LLO molecule itself.

IL-18 is first synthesized as pro-IL-18, then processed to mature form and secreted from cells in response to appropriate stimuli. To clarify which stage of IL-18 production is stimulated by LLO, we

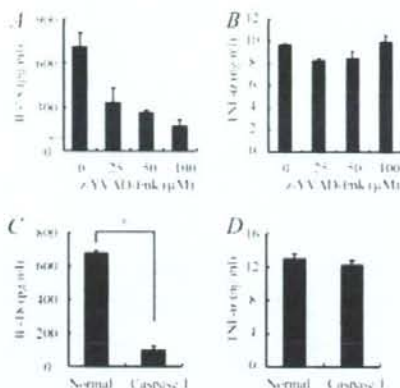

FIGURE 3. Caspase-1-dependent IL-18 production on LM infection. *A* and *B*, Adherent PECs were infected with wild-type LM. The cells were cultured for an additional 24 h in the presence of gentamicin with or without z-YVAD-fmk. *C* and *D*, Adherent PECs from normal and caspase-1 knockout mice were infected with wild-type LM. Cells were cultured for 24 h in the presence of gentamicin and then the culture supernatants were collected. The amounts of cytokines were determined by ELISA. Data represent the mean of triplicate assays and SD. Similar results were obtained in three independent experiments. *, $p < 0.01$.

next analyzed the levels of IL-18 mRNA in macrophages infected with each mutant strain. In contrast to the significant difference in the production of mature IL-18 shown above, there was no significant difference in the ability to induce the expression of IL-18 among all of the LM strains used (Fig. 2). Therefore the difference appeared to be dependent on the process that follows IL-18 gene expression.

Involvement of caspase-1 in the production of IL-18 induced by LM infection

Caspase-1, known also as IL-1β-converting enzyme, cleaves pro-IL-18 into mature form (20, 26–28). When macrophages were pretreated with z-YVAD-fmk, a caspase-1-specific inhibitor, IL-18 production induced by wild-type LM was decreased in a dose-dependent manner (Fig. 3*A*). A nonspecific inhibitory effect of z-YVAD-fmk was ruled out, because the same concentration of this inhibitor did not affect the production of TNF-α (Fig. 3*B*). To further confirm the involvement of caspase-1 in LLO-dependent IL-18 maturation, macrophages from caspase-1-deficient mice were infected with wild-type LM and the levels of IL-18 in culture supernatants were then compared with those in macrophages from normal mice. As expected, there was a significant reduction of IL-18 production in caspase-1-deficient macrophages, whereas the same cells produced TNF-α at a level comparable to that produced by the cells from normal mice (Fig. 3, *C* and *D*).

Caspase-1 activation in macrophages infected with LM strains

On the basis of the above result, we have compared the expression and processing of caspase-1 between recombinant LM mutants producing either LLO or ILO. Quantitative real-time RT-PCR detection of caspase-1 mRNA in the peritoneal macrophages showed a constitutive expression even in the absence of infection, and there was no significant level of further induction by infection with any strain (Fig. 4*A*). Caspase-1 synthesized as an immature form is converted into active caspase-1 composed of p10 and p20 fragments by proteolytic cleavage and is released from the cells soon after the conversion (29, 30). To detect the active form of caspase-1 efficiently, we enriched caspase-1 in the culture supernatants by immunoprecipitation using anti-caspase-1 Ab, according to our own modification of the method reported recently (29). LLO-producing Δ*hly*::*hly* strongly induced the processing of immature caspase-1 as determined by the detection of a p10 fragment (Fig. 4*B*). A faint band could be observed also in lysate of macrophages infected with Δ*hly*::*ilo*; however, it never reached to the level observed by Δ*hly*::*hly* during 20 h of cultivation. Following the detection of the p10 fragment in the cell lysate, the activated form of caspase-1 became detectable only in the supernatant from the culture of macrophages infected with Δ*hly*::*hly* (Fig. 4*B*, *bottom*). The very faint level of p10 fragment induced by Δ*hly*::*ilo* infection never increased even if the culture time was extended to

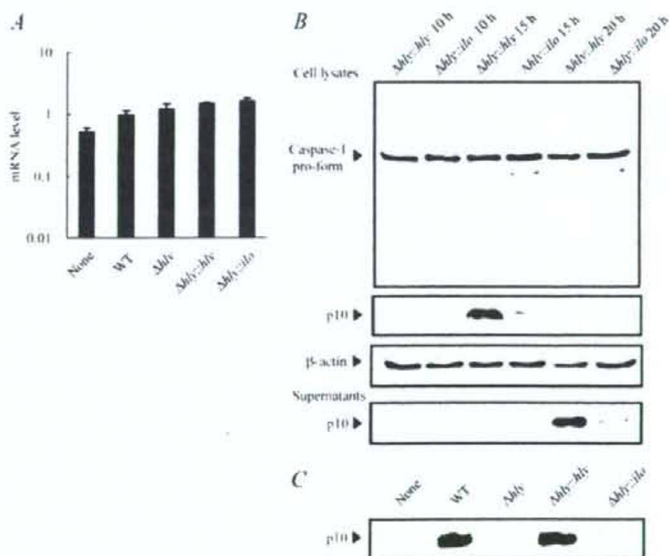

FIGURE 4. Expression and activation of caspase-1 in macrophages infected with LM strains. *A*, Adherent PECs were infected with each LM strain. The cells were cultured for an additional 5 h in the presence of gentamicin. Total RNA was extracted and subjected to quantitative real-time RT-PCR for detection of mRNA for caspase-1. Data represent the mean of triplicate assays and SD. *B*, The cells were cultured for an indicated time, and the culture supernatants were then collected. Adherent cells were lysed with 1% Nonidet P-40 lysis buffer. Active caspase-1 in the supernatants was immunoprecipitated and immunoblotted using a rabbit anti-caspase-1 Ab. *C*, After cultivation for 20 h, the active form of caspase-1 in the supernatants of the cells infected with each LM strain was detected. WT, Wild type.

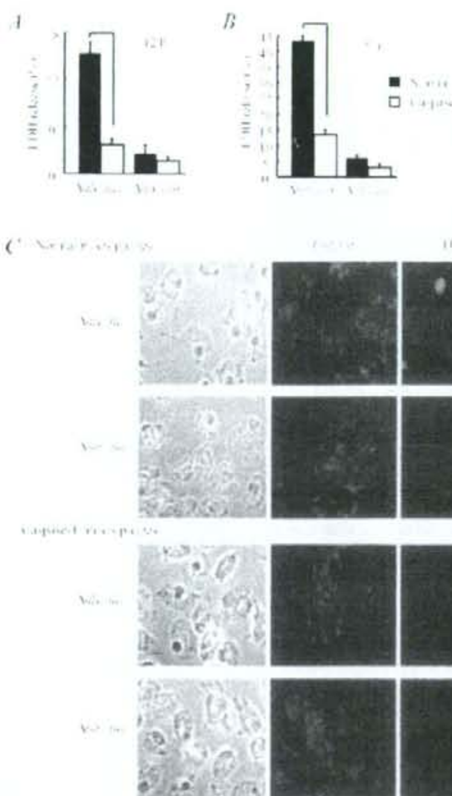

FIGURE 5. Detection of caspase-1-dependent cell death in macrophages infected with *Δhly::hly* or *Δhly::ilo*. *A* and *B*, Adherent PECs were infected with *Δhly::hly* or *Δhly::ilo*. The cells were cultured for an indicated time in the presence of gentamicin. The culture supernatants were collected and the LDH activity was assayed. Data represent the mean of triplicate assays and SD. Similar results were obtained in three independent experiments. *, $p < 0.01$. *C*, The cells were cultured for 21 h and visualized by staining for *Listeria* (red), fragmented DNA (green), and total nucleus (blue). DAPI, 4′,6-Diamidino-2-phenylindole.

30 h or of the MOI was increased to 10 (data not shown). The active form of caspase-1 was also detected in the supernatant from wild-type LM-infected macrophages, but not from *Δhly*-infected cells (Fig. 4*C*). These results clearly indicated that caspase-1 activation induced upon infection with LM is dependent on not only the entry of bacteria into the macrophage cytoplasm but also the LLO molecule itself.

Caspase-1-dependent cell death of macrophages infected with LM strains

It has been reported that LM causes an unique type of cell death of the infected host macrophages, which is distinguished as pyroptosis from other forms of programmed cell death by its requirement of caspase-1 activation and the loss of plasma membrane integrity (21, 31). Other intracellular bacteria, *Salmonella* and *Shigella*, also induce pyroptosis accompanied by the release of LDH in a caspase-1-dependent manner only at an early phase of cell death, but not in late phases (32, 33). To know whether caspase-1-dependent events other than IL-18 maturation occur during infection with LLO-producing LM, we next determined LDH released from infected macrophages. The amount of LDH released from normal macrophages was higher than that from caspase-1-deficient macrophages when infection was done with *Δhly::hly* (Fig. 5, *A* and *B*). In contrast, *Δhly::ilo* induced a significantly lower level of LDH release compared with *Δhly::hly*, and there was no significant difference in the amount of LDH released after infection with *Δhly::ilo* between normal and caspase-1-deficient macrophages. Upon pyroptotic cell death, DNA cleavage occurred and could be detected by the TUNEL method (31, 34). Therefore we also tried to determine the level of pyroptosis by the visualization of fragmented DNA in *Listeria*-infected cells. As shown in Fig. 5*C* and Table I, TUNEL-positive cells were observed frequently in normal macrophages infected with *Δhly::hly* but not in those infected with *Δhly::ilo*. The DNA fragmentation induced by *Δhly::hly* was caspase-1 dependent, because the number of TUNEL-positive cells was fewer and similar to the control level in the absence of caspase-1. These data indicated that LLO-producing LM, but not ILO-producing LM, induces a caspase-1-dependent pyroptosis in addition to the processing of IL-18 and supported our finding that the delivery of bacteria into the cytoplasm is not sufficient but that the presence of LLO is required for the induction of caspase-1 activation upon LM infection.

LLO-dependent caspase-1 activation does not require TLR4-mediated signaling

Because LLO has been reported as a ligand for TLR4 when added from outside of the cells (35), we examined the involvement of TLR4 in the production of IL-18 and the activation of caspase-1 in response to wild-type LM. Macrophages obtained from normal or TLR4-deficient mice were infected with LLO-producing wild-type LM and the culture supernatants were then subjected to cytokine assays. As shown in Fig. 6, *A–D*, the levels of cytokines, including IL-18, in supernatants of LM-infected TLR4-deficient macrophages were comparable to those of infected macrophages from normal mice, indicating that TLR4 is not involved in the production of IL-18 and other cytokines in response to the infection with LM. Furthermore, wild-type LM induced similar levels of caspase-1 activation in normal and TLR4-deficient macrophages

Table I. *Percentage of TUNEL-positive (TUNEL⁺) cells in Listeria-infected cells or total cells*

Macrophage	Bacteria	TUNEL⁺/*Listeria*-Infected Cells (%) (Green/Red⁺ Cell × 100)[a]	TUNEL⁺/Total Cells (%) (Green/Blue × 100)[a]
Normal	None		1.95 ± 0.40
	Δ*hly*::*hly*	28.80 ± 1.83	15.83 ± 0.76
	Δ*hly*::*ilo*	4.60 ± 0.72	2.06 ± 0.20
Caspase-1⁻	None		1.95 ± 0.20
	Δ*hly*::*hly*	5.73 ± 0.55	3.30 ± 0.36
	Δ*hly*::*ilo*	7.10 ± 1.41	3.06 ± 0.65

[a] Value represents means and SDs of three independent wells. Three hundred cells were examined in each well.

(Fig. 6*E*). From these results, it is indicated that the LLO-dependent caspase-1 activation and the subsequent production of mature IL-18 are not due to the recognition of LLO by TLR4.

Detection of the domain of LLO responsible for caspase-1 activation

To further confirm the involvement of LLO in caspase-1 activation induced upon LM infection, we constructed a Δ*hly*::*ilo* strain additionally expressing the full-length LLO by using the pAT28 expression vector. The activation of caspase-1 was observed when macrophages were infected with the Δ*hly*::*ilo* carrying full-length LLO expression vector, whereas Δ*hly*::*ilo* transformed with empty vector could not induce caspase-1 activation (Fig. 7*B*). Among the four domains comprising the whole LLO molecule, domain 4 is known as a cholesterol-binding domain and contains Trp-rich undecapeptide, which is highly conserved among cholesterol-dependent cytolysins and essential for the binding to membrane cholesterol (36). It has been previously reported that the domain 1–3 molecule of truncated LLO completely loses its cytolytic activity and the substitution of the third Trp of the undecapeptide (amino acid residue 492 of LLO holotoxin) with Ala severely attenuates the cytolytic activity of LLO (36, 37). Therefore, we next constructed Δ*hly*::*ilo* strains carrying each vector for the expression of LLO W492A or domain 1–3 of LLO to know whether cholesterol-binding and subsequent cytolysis by this cytolysin is required for the activation of caspase-1 in macrophages infected with LM. The production of different LLO molecules encoded by each plasmid was confirmed by Western blotting using an anti-LLO polyclonal Ab (Fig. 7*A*). The impaired ability of Δ*hly*::*ilo* in caspase-1 activation was restored by the introduction of a plasmid harboring the gene for full-length LLO, and such an effect was not affected even by the elimination of cytolytic activity as clearly shown by LLO W492A or LLO domain 1–3 (Fig. 7*B*). By contrast, complementation with a plasmid harboring the domain 4 of LLO, a molecule capable of cholesterol binding without cytolytic activity (38), never resulted in the acquisition of the ability for caspase-1 activation (Fig. 7*B*). As there was no significant difference in the level

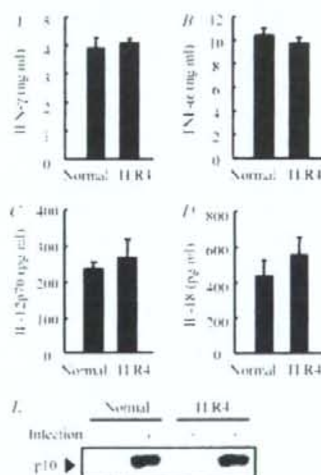

FIGURE 6. Cytokine production and caspase-1 activation in TLR4 knockout macrophages. *A–D*, Whole PECs (*A*) and adherent PECs (*B–D*) from normal and TLR4 knockout mice were infected with wild-type LM. Cells were cultured for 24 h in the presence of gentamicin and then the culture supernatant was collected. The amount of each cytokine was determined by using ELISA specific for each cytokine. Data represent the mean of triplicate assays and SD. Similar results were obtained in three independent experiments. *, $p < 0.01$. *E*, Adherent PECs from normal and TLR4 knockout mice were infected with wild-type LM. Cells were cultured for 20 h in the presence of gentamicin and the culture supernatants were collected. Active caspase-1 in the supernatants was immunoprecipitated and immunoblotted using a rabbit anti-caspase-1 Ab.

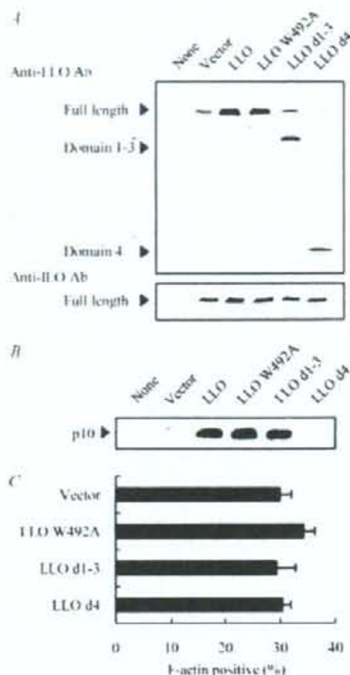

FIGURE 7. Detection of the domain (d) of LLO responsible for caspase-1 activation. *A*, Culture supernatants of Δ*hly*::*ilo* strains transformed with each LLO-expressing vector were applied to SDS-PAGE and subsequent Western blotting using anti-LLO Ab (*upper panel*) or anti-ILO Ab (*lower panel*). *B*, Adherent PECs were infected with each Δ*hly*::*ilo* strain, cultured for 20 h in the presence of gentamicin and spectinomycin (250 μg/ml), and culture supernatants were then collected. Active caspase-1 in the supernatants was immunoprecipitated and immunoblotted using a rabbit anti-caspase-1 Ab. *C*, Adherent PECs were infected with each Δ*hly*::*ilo* strain, cultured for 3 h in the presence of gentamicin and spectinomycin, and then bacteria and F-actin were stained and 300 bacteria were counted. The percentage of bacteria positive for associating F-actin was calculated for each strain. The filled bars represent the mean of three independent wells, and the error bars indicate the SD.

of F-actin-positive bacteria inside macrophages among these plasmid-carrying *Δhly::ilo* strains (Fig. 7*C*), it was clearly indicated that domain 1–3 is the region responsible for the activation of caspase-1 in infected macrophages.

Discussion

We previously showed that an entry of bacteria into the cytoplasm was not sufficient for the IFN-γ response of mice to LLO-producing LM by both in vitro and in vivo experiments using two isogenic strains that differed only in LLO and ILO (11). In this study, we examined the difference between *Δhly::hly* and *Δhly::ilo* in the induction of IL-12 and IL-18, the two major IFN-γ-inducing cytokines, to elucidate the mechanism of LLO-dependent IFN-γ response to LM. It was noteworthy that LLO-producing LM, but not ILO-producing LM, strongly induced the production of IL-18. Because mRNA expression did not depend exclusively on LLO, we next examined the activation of caspase-1, which is essential for the maturation and secretion of biologically active IL-18. It was strongly suggested that the dependence of the IFN-γ response on LLO is due to the LLO-dependent induction of caspase-1 activation and subsequent IL-18 production and that the IL-18 response is dependent on not only the entry of bacteria into the macrophage cytoplasm but probably also on the distinct activity of LLO as a signaling ligand.

On the basis of a previous report (18) and our own similar observation (not shown) that IL-12 is not induced in mice deficient for MyD88, an adaptor molecule of almost all TLRs, upon infection with LM, it is clear that TLR-dependent recognition of bacterial ligands is essentially required for the induction of IL-12 production. By contrast, production of IL-18 is reported to be induced regardless of the absence of MyD88 (39). Our present finding that TLR4, a recognition receptor for LLO, was not involved in the activation of caspase-1 and production of IL-18 in macrophages infected with LM is consistent with these previous observations. Generally, IL-18 is constitutively expressed, and the produced pro-IL-18 remains inside the cells until being cleaved by activated caspase-1. Therefore both MyD88-dependent production of IL-12 and TLR4- and MyD88-independent cleavage of pro-IL-18 are likely the key processes for the induction of IFN-γ upon LM infection. Some distinct activity of LLO appeared to be involved in the latter process that cannot be induced by ILO.

Although the involvement of LLO in caspase-1-mediated IL-18 induction upon infection with LM was revealed clearly in this study, the mechanism remains to be elucidated. One possibility to be considered is the difference in the cytotoxic effect between LLO and ILO. These two cytolysins are highly homologous and exhibited a quite similar function regarding the contribution to the escape of *Listeria* from phagosome into the cytoplasm. After the escape of bacteria from the hazardous phagosome by means of cytolysin into the cytosol, a nutrient-rich niche for multiplication, it is important for the cytosolic bacteria to minimize further cytolytic activity to prevent host cell damage. For that, two mechanisms have been proposed: the dependency of cytolytic activity on acidic pH (40) and the presence of an N-terminal Pro-Glu-Ser-Thr (PEST)-like sequence, which is thought to target for phosphorylation and/or degradation in eukaryote cells (41). Although both LLO and ILO exhibited a similar optimal pH for cytolytic activity (42), the hemolytic activity of recombinant ILO was relatively higher than that of recombinant LLO (17). Besides, the sequence analysis of the *ilo* gene revealed the absence of the PEST-like sequence that is present in LLO (data not shown). In the present experimental results, the level of cytolysis induced by ILO-producing LM was marginal and rather lower than that induced by LLO-producing LM in caspase-1-deficient cells (Fig. 5). Moreover, these LM strains induced similar levels of TNF-α and IL-12 production by macrophages (Fig. 1). Therefore the above possibility could be ruled out, and the difference in the ability to induce IL-18 between LLO-producing LM and ILO-producing LM should be explained by other reasons. Construction of the *Δhly* strains complemented with genes encoding chimeric proteins between LLO and ILO and recombinant strains expressing cytolysins mutated for PEST-like sequence, are under way, and the molecular basis for the LLO-dependent activation of caspase-1 should be clarified in the near future.

From our results using LLO-producing LM and ILO-producing LM, there may be two major possibilities as follows: 1) LLO activates some signaling pathway that leads to caspase-1 activation without the participation of any other ligands; and 2) LLO induces the activation of caspase-1 in cooperation with other bacterial ligands or merely enhances that induced by other bacterial ligands. If either of these possibilities is the case, ILO itself must have no or less ability to induce caspase-1 activation compared with LLO. In our previous study using recombinant LLO and ILO, it was shown that ILO is far less capable of inducing cytokines than LLO when added from outside of the cells in vitro (17). Therefore our assumption is that there is some molecular structure in LLO that is exclusively important for caspase-1 activation in the cytosol of macrophages. Indeed, our results using *Δhly::ilo* strains additionally expressing the recombinant proteins of LLO molecule (Fig. 7) clearly indicated that domain 1–3 is the region responsible for such an activity after the escape of bacteria into the cytosolic space and also that the ability of the LLO molecule for membrane binding or membrane damage is not involved in the activation of caspase-1. In a further study, experiments on a transfection of macrophages with LLO- or ILO-expressing vector or an intracytosolic injection of recombinant cytolysin are to be conducted.

Compared with virulence gene expression in broth-cultured LM, the expression of LLO is believed to be up-regulated inside macrophages (43). It is therefore reasonable that some cytoplasmic sensor molecule, rather than a cell surface receptor, recognizes LLO, resulting in caspase-1 activation. It has been reported that LLO is recognized by TLR4 and activates a signaling pathway downstream of TLR4 (35). Moreover, recombinant LLO protein induced the production of various cytokines by splenocytes or PEC cultures in a TLR4-dependent manner, whereas recombinant ILO protein did not induce their production (17, 35). However, our present results indicated that TLR4 was not involved in the production of IL-18 and the activation of caspase-1 in macrophages infected with LM (Fig. 6). Several recent reports have shown that some bacterial ligands are recognized not only by TLRs but also by NLRs such as Ipaf/CARD12 and Nalp3/cryopyrin/Pypaf1, which are cytoplasmic proteins containing a leucine-rich repeat domain, a nucleotide-binding domain, and each signaling domain. Flagellin from *Salmonella* or *Legionella*, which is known as a TLR5 ligand (44), induces caspase-1 activation through an Ipaf-dependent pathway when it is in the cytoplasm (45–47). The small antiviral compounds imiquimod and R-848, which are TLR7 ligands (48), are also known to induce the activation of caspase-1 in a Nalp3-dependent manner (49). Moreover, Nalp1b, an NLR protein, is involved in the caspase-1-dependent cell death of mouse macrophages induced by lethal toxin, which is a protein toxin produced by *Bacillus anthracis*, suggesting that lethal toxin induces the activation of caspase-1 through Nalp1b directly or indirectly (50). In the case of infection with *Listeria*, caspase-1 activation in macrophages

is reported to depend upon an apoptosis-associated speck-like protein containing a C-terminal caspase recruitment domain, ASC, an adaptor molecule that links upstream NLRs to caspase-1 (20). Furthermore, it is reported that potassium efflux and the P2X7 receptor are not required for caspase-1 activation induced by *Listeria* infection (22). On the basis of these recent findings, it is highly possible that LM-induced caspase-1 activation is triggered by the recognition of cytosolic LLO by some NLR protein. The experiments in this line will be conducted also by using recombinant strains under construction.

In conclusion, this study has clearly indicated that LM-induced IFN-γ production in mice is ascribed mainly to the presence of LLO due to a distinct activity of inducing the activation of caspase-1 after evasion into the cytosolic space of infected macrophages.

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Disclosures

The authors have no financial conflict of interest.

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A novel method for simple detection of mutations conferring drug resistance in *Mycobacterium leprae*, based on a DNA microarray, and its applicability in developing countries

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A simple method to detect mutations in the genome of *Mycobacterium leprae* that confer resistance to key drugs for leprosy was exploited on the basis of a reverse hybridization system. A series of oligonucleotide probes corresponding to each mutation in the *folP1*, *rpoB* and *gyrA* genes for dapsone, rifampicin and ofloxacin resistance, respectively, were selected and fixed on a glass slide as capture probes, to develop a DNA microarray termed the leprosy drug susceptibility-DNA microarray (LDS-DA). Mutations in clinical isolates of *M. leprae* were successfully identified by the LDS-DA. Feasibility studies were conducted to evaluate the performance of the LDS-DA in two developing countries, Myanmar and the Philippines. The high concordance of the results obtained by this method with the results of nucleotide sequencing strongly supports the applicability of the LDS-DA as a drug susceptibility test in place of sequencing, a time-consuming and costly procedure. This is a rapid and simple method for the simultaneous susceptibility testing of three front-line drugs for leprosy, and solves the problems of previously reported methods.

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INTRODUCTION

The current strategy for leprosy control relies mainly on multidrug therapy (MDT) (WHO, 1998). However, cases of leprosy caused by drug-resistant *Mycobacterium leprae* have been documented as the result of therapeutic failure (Cambau *et al.*, 2001; Maeda *et al.*, 2001; Matsuoka *et al.*, 2000, 2003). Although information on the drug susceptibility of clinical isolates contributes to the better outcome of treatment, susceptibility testing has rarely been done because of its difficulty. Antibiotic susceptibility testing of *M. leprae* still relies on a time-consuming method based on the growth of bacteria in mouse footpads (Shepard, 1960), which takes up to 12 months to give a result. This has hindered the comprehensive surveillance that would offer useful information to evaluate the efficacy of MDT and to prevent the spread of drug-resistant strains. Recent advances in the molecular biology of drug-resistant *M. leprae* have enabled the development of drug susceptibility tests for key component drugs of MDT, by the detection of relevant gene mutations that confer resistance (Williams & Gillis, 2004). The molecular mechanism of rifampicin resistance was first demonstrated in *Escherichia coli* and thereafter in *M. leprae* (Honoré & Cole, 1993). Rifampicin resistance is strongly correlated with mutations in the *rpoB* gene, encoding the β subunit of RNA polymerase (Honoré & Cole, 1993; Honoré *et al.*, 1993; Williams *et al.*, 1994, 2001; Matsuoka *et al.*, 2000, 2003; Cambau *et al.*, 2001; Maeda *et al.*, 2001; Zhang *et al.*, 2004). Resistance to fluoroquinolones has been proved to correlate with

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Abbreviations: BI, bacterial index; DRDR, drug-resistance-determining region; LDS-DA, leprosy drug susceptibility-DNA microarray; MDT, multidrug therapy.

mutations in the *gyrA* gene, encoding the A subunit of DNA gyrase in *M. leprae* (Cambau *et al.*, 1997; Matsuoka *et al.*, 2000; Cambau *et al.*, 2001), as in many other bacteria. In addition, mutations in the *folP1* gene, encoding dihydrofolate synthetase, have been shown to be responsible for dapsone resistance (Kai *et al.*, 1999; Matsuoka *et al.*, 2000, 2003, 2007; Williams *et al.*, 2000; Lee *et al.*, 2001; Maeda *et al.*, 2001; Cambau *et al.*, 2006). The prevalence of drug resistance in selected areas was surveyed through the application of molecular analysis to detect mutations conferring drug resistance (Matsuoka *et al.*, 2007). Analysis of mutations is generally performed by sequencing the target genomic region, amplified by PCR, although the implementation of sequencing is not easy in many developing countries. Therefore, a simple and rapid method to detect mutations conferring drug resistance has been long awaited. In the current study, a DNA microarray method was developed and the applicability of this system was evaluated in Myanmar and the Philippines.

METHODS

Design of capture probes. Mutant nucleotide sequences conferring resistance to dapsone, rifampicin and ofloxacin and their corresponding wild-type sequences in *Mycobacterium leprae* (Table 1) were employed in this study. Nearly all the drug-resistant strains of *M. leprae* reported so far are covered by the mutations selected. Capture oligonucleotide probes (14- to 18-mer) for the detection of the mutations were designed according to these data. Optimal sequences of oligonucleotides corresponding to each missense mutation were designed empirically as shown in Fig. 1(a). The array of capture oligonucleotide probes was covalently bound to the surface of a glass slide coated with polycarbodiimide and the resulting DNA microarray was designated the leprosy drug susceptibility-DNA microarray (LDS-DA), as shown in Fig. 1(b).

Amplification of three target gene fragments. Target regions of *folP1* (accession no. AL 583917, gene ML583917), *rpoB* (accession no. AL583923, gene ML1891) and *gyrA* (accession no. AL583917, gene ML0006) were simultaneously amplified with three primer pairs in one PCR. The sequences of the primers are listed in Table 2. PCR was carried out using the G mixture of the FailSafe PCR System (EPICENTRE) in a volume of 25 µl with 1 µM of each primer. Cycling conditions began with an initial incubation at 94 °C for 4 min, followed by 40 cycles of annealing at 58 °C for 30 s, extension at 72 °C for 30 s, and denaturation at 94 °C for 30 s. Finally, incomplete PCR products were extended for 5 min at 72 °C. The amplified DNA fragments were confirmed by gel electrophoresis through 4.0% Metaphor Agarose (FMC Corp.) in TBE (Tris/borate/EDTA, pH 8.0) buffer.

LDS-DA assay. A 2 µl aliquot of the resulting PCR mixture was mixed with 38 µl UniHyb Hybridization Solution (TeleChem International), heat denatured at 98 °C for 5 min and quickly chilled. The solution was then applied to the LDS-DA and incubated at 42 °C for 60 min followed by stringent washing with 50 µl washing solution (3 M tetramethylammonium chloride; Sigma-Aldrich) at 47 °C for 60 min. The biotin-labelled DNA fragments hybridizing to the capture probes on the LDS-DA were detected by avidin-biotin-horseradish peroxidase complex (VECTASTAIN Elite ABC kit, Vector

Table 1. Missense mutations associated with drug resistance in *M. leprae*

Drug	Gene	Codon no.	Susceptible		Resistant		References†
			Codon	AA*	Codon	AA*	
Dapsone	*folP1*	53	ACC	Thr	GCC	Ala	1, 2, 3
					GTC	Val	4
					ATC	Ile	2, 3, 5, 6
					AGG	Arg	3
					AGA	Arg	4
		55	CCC	Pro	TCC	Ser	1, 7
					CGC	Arg	1, 3
					CTC	Leu	2, 3, 8
Rifampicin	*rpoB*	407	CAG	Gln	GTG	Val	9
		410	GAT	Asp	AAT	Asn	2
					TAT	Tyr	8
		420	CAC	His	TAC	Tyr	2
					GAC	Asp	9, 10
		425	TCG	Ser	ATG	Met	9, 10
					TTG	Leu	2, 9
					TTC	Phe	11, 2, 6, 7, 3
		427	CTG	Leu	CCG	Pro	2
Ofloxacin	*gyrA*	89	GGC	Gly	TGC	Cys	2
		91	GCA	Ala	GTA	Val	12, 2, 6

*AA, amino acid.

†1, Lee *et al.* (2001); 2, Maeda *et al.* (2001); 3, Williams *et al.* (1994); 4, Matsuoka *et al.* (2007); 5, Kai *et al.* (1999); 6, Matsuoka *et al.* (2000); 7, Matsuoka *et al.* (2003); 8, Zhang *et al.* (2004); 9, Cambau *et al.* (2001); 10, Honore & Cole (1993); 11, Honore *et al.* (1993); 12, Cambau *et al.* (1997).

(a)

Drug	Gene	Codon no.	Capture probes for wild-type	Capture probes for mutants
Dapsone	*folP1*	53	FW1:GTTGGCGAATCGACCCGG	FM1:TGGCGAATCGGCCCGG
				FM2:TGGCGAATCGGTCCGG
				FM3:TGGCGAATCGATCCGG
				FM4:GGCGAATCAGGCGG
				FM5:TGGCGAATCGAGACGG
		55	FW2:[illegible]CCATTA	FM6:[illegible]
				FM7:[illegible]
				FM8:[illegible]
Rifampicin	*rpoB*	407	RW1:AGCTGTCGGCAGTTCATG	RM1:AGCTGTCGGTGTTCAT
		410	RW2:TTCATGGACCAGAA	RM2:TTCATGAATCAGAACAA
				RM3:TTCATGTATCAGAACAA
		420	RW3:CCTGACCCACAAGCGC	RM4:GCCTGACCTACAAGCCC
				RM5:GCCTGACCCACAAGCCC
		425	RW4:GCCGACTGTCGGCGCTG	RM6:GCCGACTGATGGCGC
				RM7:GCCGACTGTTGGCG
				RM8:GCCGACTGTTCGCG
		427	RW5:GCGCTGGGGGCCCGGGTG	RM9:GGCGCCGGGGCCCGGGTG
Ofloxacin	*gyrA*	89	GW1:ATCCGGCACGGCGACGCA	GM1:ATCCGGCACTGCGACGCA
		91	GW2:CGGCGACGCATCGATCT	GM2:CGGCGACGTATCGACTT
Positive hybridization control in *gyrA*			GP:CGACCCTAGCCACGCTAA	
Negative hybridization control in *gyrA*			GN:CGACCCTCATCACGCTAA	

(b)

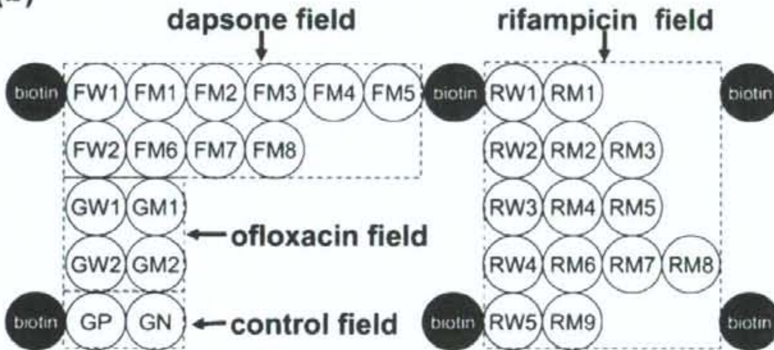

Fig. 1. Development of the LDS-DA. (a) The oligonucleotide sequences used in the test. Codons relating to drug resistance are underlined. Oligonucleotide GP, positive control for PCR amplification and hybridization; GN, negative control for hybridization. (b) Schematic representation of the array of oligonucleotides on the LDS-DA. Black circles represent spots with biotin as landmarks for conjugate reaction control; grey circles are the wild-type spots; white circles are the mutant spots. The region with oligonucleotides designated FW- and FM- is for dapsone resistance detection (the dapsone field); the region designated GW- and GM- is the ofloxacin field; the region designated GP- and GN- is the control field; and the region designated RW- and RM- is the rifampicin field.

Laboratories) and then visualized by TMB peroxidase substrate (Vector Laboratories). The resulting spot patterns were recorded by a conventional scanner and a computer. Only the results of the LDS-DA with proper signals on both positive and negative control spots (GP and GN in Fig. 1) were used for further analysis. The colour intensity of each spot in a row (covering the same region of each gene) was examined. The spot with the highest colour intensity was considered to reflect the sequence of the gene fragment in the sample.

Evaluation of the LDS-DA with clinical specimens. The LDS-DA system was transferred to laboratories in the Department of Medical Research in Yangon, Myanmar, and in the Leonard Wood Memorial in Cebu, the Philippines, and was evaluated on 63 and 73 clinical specimens, respectively, in these laboratories. A majority of the samples in this study had been examined previously (Matsuoka *et al.*, 2007). Of the 63 samples in Myanmar, 44 were from new cases and 19 were from patients with relapse. Of the 19 relapsed patients, one patient had received monotherapy with dapsone for 4 years followed by monotherapy with rifampicin for 4 years, while the other 18 patients had been treated with the standard MDT regimen for multibacillary leprosy. Samples from the Philippines included 64 from new cases and nine from relapsed cases. Of the nine relapsed

Table 2. Sequences of oligonucleotide primers for *M. leprae*

Gene	Primer	Sequence (5′–3′)	PCR products (bp)
folP1	MLfolP1DA-Ft;	GTGAGTTTGGCGCCAGTGCA	119
	MLfolP1DA-RB;	Biotin-GCAAGTTCTTTTACGACAGG	
rpoB	MLrpoBDA-F;	TCGCCGCTATCAAGGAATTC	127
	MLrpoBDA-RB;	Biotin-TCACGCGACAAACCACCCGG	
gyrA	MLgyrADA-F;	TGAGACTCCGGTTTCCGCCC	139
	MLgyrADA-RB;	Biotin-CAGCGACCACGGCTGCGCC	

cases, three patients had been treated with the WHO MDT regimen for 2 years and the other six patients had received dapsone monotherapy or combined treatment with clofazimine and rifampicin. All cases had a positive bacterial index (BI) and were therefore, by definition, multibacillary. Genomic DNA templates were prepared as described previously (Matsuoka *et al.* 2005, 2007). Briefly, slit-skin smear specimens were collected from the skin lesions of patients in the same manner as the routine procedure for BI determination. The bacilli were washed out from the blade into 70% ethanol and collected as a pellet by centrifugation at 10 000 **g** for 20 min. Genomic DNA templates for PCR were prepared by treatment with a lysis buffer as described elsewhere (de Wit *et al.*, 1991). The LDS-DA assays were performed as described above and the results were translated into nucleotide sequences according to the positions of the spots for comparison with the sequence data.

Nucleotide sequencing. To confirm and verify the results obtained by the LDS-DA method, nucleotide sequences of PCR products were determined with the BigDye Terminator v1.1 Cycle Sequencing kit (Applied Biosystems) using the same primers for PCR amplification with an ABI310 genetic analyser.

Ethical approval and consent. The study was approved by the institutional ethics committee of the National Institute of Infectious Diseases, Japan, and two local institutional review boards. Bacterial samples were collected after informed consent was obtained.

RESULTS

Development of a DNA microarray for drug susceptibility testing of *M. leprae*

The target regions of the genes with expected length, 119 bp for *folP1*, 127 bp for *rpoB* and 139 bp for *gyrA*, were amplified simultaneously by mutiplex PCR as shown in Fig. 2. Several oligonucleotides corresponding to each of the wild-type and mutant sequences of *folP1*, *rpoB* and *gyrA* were synthesized, spotted on a glass slide and examined for hybridization with amplicons from the multiplex PCR. The best oligonucleotides, which hybridized with corresponding PCR products without reacting with others, were selected. A DNA microarray with selected oligonucleotides was established as presented in Fig. 1 and designated LDS-DA. The performance of the LDS-DA was examined using PCR products from *M. leprae* isolates and artificially produced DNA fragments with known mutations. PCR products containing the drug-resistance-determining region (DRDR) for each gene were obtained by multiplex PCR (Fig. 2). Fig. 3 shows the hybridization patterns obtained from isolates grown in nude mice footpads, Thai-

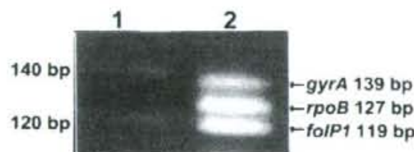

Fig. 2. Electrophoretic pattern obtained by multiplex PCR for *folP1*, *rpoB* and *gyrA*. Lane 1, 20 bp ladder size markers; lane 2, PCR products.

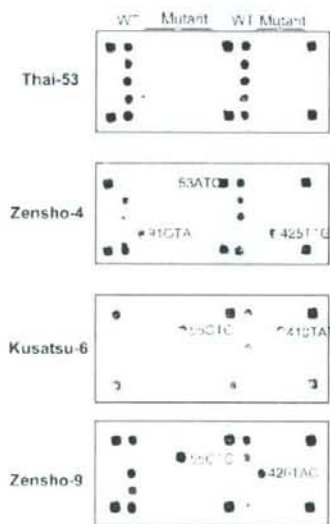

Fig. 3. Signals obtained by the LDS-DA with: a susceptible strain, Thai-53; strain Zensho-4, with mutation from ACC to ATC at codon 53 in the *folP1* gene, from TCG to TTG at codon 425 in the *rpoB* gene and from GCA to GTA at codon 91 in the *gyrA* gene; strain Kusatsu-6, with mutation from CCC to CTC at codon 55 in the *folP1* gene and from GAT to TAT at codon 410 in the *rpoB* gene; and strain Zensho-9, with mutation from CCC to CTC at codon 55 in the *folP1* gene and from CAC to TAC at codon 420 in the *rpoB* gene.

53, Zensho-4 (Matsuoka *et al.*, 2000) and two other strains with known nucleotide mutations (Zhang *et al.*, 2004; Maeda *et al.*, 2001). In Thai-53, which is susceptible to dapsone, rifampicin and ofloxacin, positive signals were observed on all of the wild-type spots. In contrast, the highest colour intensity was seen on the spot with the mutant oligonucleotide in drug-resistant *M. leprae*. In Zensho-4, with a three-drug-resistant phenotype, three positive signals shifted from the wild-type to the mutant spots. In the dapsone field, a mutation at codon 53 was identified by a positive signal on spot FM3 instead of FW1. In the ofloxacin and rifampicin fields, similar events were observed. Spots corresponding to mutant-type and wild-type sequences were also found in the other two isolates. Likewise, all the spots with mutant oligonucleotides were verified as to their proper reactivity with the PCR products carrying corresponding known mutations (data not shown).

Evaluation of the LDS-DA system in two countries with high leprosy prevalence

The LDS-DA system was successfully transferred to a laboratory in Yangon, Myanmar, and a laboratory in Cebu, the Philippines. The BI values of the samples from Myanmar varied from 1 to 6. Most were more than 3. Almost all samples from the Philippines showed a BI of more than 4, with a few samples of BI 2. Positive PCR results were obtained even from samples with a BI of 1, although it was usually hard to obtain good results from PCR and colouring from samples with a BI of less than 3. One of the relapsed cases from Myanmar harboured *M. leprae* with mutations CCC to CGC at position 55 in the *folP1* gene and TCG to ATG at position 425 in the *rpoB* gene. One resistant isolate with the mutation ACC to GCC at position 53 in the *folP1* and another isolate with the mutation GAT to TAT at position 410 in the *rpoB* gene were new cases. In the samples from the Philippines, three *M. leprae* with mutations in the *folP1* gene, CCC to CTC and CCC to TCC at position 55, were from relapsed cases. Two resistant isolates with mutation CCC to CGT at position 55 in the *folP1* were from new cases. The results obtained by the LDS-DA system in these laboratories were compared with the nucleotide sequences of the corresponding genes, as shown in Table 3. All the samples possessing wild-type sequences were judged to be wild-type by both the LDS-DA and sequencing. Concordant results were also observed with seven specimens carrying mutations, five in Myanmar and two in the Philippines. Two unclear results were obtained in *folP1* in Myanmar and one in *rpoB* in the Philippines. In these three samples, the signals were not strong enough to be judged. In the row of codon 55 in *folP1*, no signal was observed with two specimens in the Philippines. Overall, the concordance between the LDS-DA and sequencing results on *folP1* in Myanmar and the Philippines is 96.8 % (61/63) and 97.3 % (71/73), respectively. The LDS-DA results on *rpoB* exhibited good agreement with sequencing results, 100 % (63/63) and 98.6 % (72/73) in Myanmar and the Philippines, respectively. No discordance was found between the LDS-DA and sequencing results on *gyrA* in either country (Table 4).

DISCUSSION

Detection of drug resistance in *M. leprae* is crucial for the efficient treatment of leprosy and the prevention of the spread of drug-resistant strains. The elucidation of the genetic background of resistance by molecular methods has enabled the prediction of drug susceptibility of *M. leprae*. Drug resistance to dapsone, rifampicin and ofloxacin has evolved by mutation in the DRDR in the *folP1*, *rpoB* and *gyrA* genes respectively (Williams & Gillis, 2004). A total of

Table 3. Comparison of results obtained by the LDS-DA and sequencing on clinical specimens in Myanmar and the Philippines

Study site	Status	*folP1*			*rpoB*			*gyrA*		
		No. of specimens	LDS-DA	Sequence	No. of specimens	LDS-DA	Sequence	No. of specimens	LDS-DA	Sequence
Myanmar	Concordant	59	WT*	WT	61	WT	WT	62	WT	WT
		1	53:GCC†	53:GCC	1	410:TAT	410:TAT	1	91:GTA	91:GTA
		1	55:CGC	55:CGC	1	425:ATG	425:ATG			
	Discordant	2	Unclear‡	WT						
Philippines	Concordant	68	WT	WT	72	WT	WT	73	WT	WT
		2	55:CTC	55:CTC						
		1	55:TCC	55:TCC						
	Discordant	2	55:null§	55:CGT	1	Unclear	WT			

*Wild-type sequence.

†Codon number : codon sequence.

‡Data could not be translated because of weak signals.

§No signal was observed on the spots in raw *folP1* 55.

Table 4. Concordance of LDS-DA results with sequencing in clinical specimens in Myanmar and the Philippines

Study site	Target gene		
	folP1	*rpoB*	*gyrA*
Myanmar	61/63 (96.8 %)	63/63 (100 %)	63/63 (100 %)
The Philippines	71/73 (97.3 %)	72/73 (98.6 %)	73/73 (100 %)
Total	132/136 (97.1 %)	135/136 (99.3 %)	136/136 (100 %)

106 isolates without mutation in the *rpoB* gene and 63 isolates without mutation the *gyrA* gene were susceptible to rifampicin and ofloxacin, respectively. All isolates resistant to rifampicin or ofloxacin harboured mutations in the DRDR of *rpoB* or *gyrA*, respectively (Honoré & Cole, 1993; Honoré *et al.*, 1993; Williams *et al.*, 1994, 2001; Cambau *et al.*, 1997, 2001; Matsuoka *et al.*, 2000, 2003; Maeda *et al.*, 2001; Zhang *et al.*, 2004). Resistance of *M. leprae* to dapsone in the mouse footpad is classified into three degrees, namely, low, intermediate and high. A total of 84 isolates without mutation in the *folP1* gene were susceptible to dapsone, but one isolate was resistant with intermediate degree and five isolates were resistant with low degree (Cambau *et al.*, 2006). On the other hand, a total of 24 isolates resistant to dapsone with high or intermediate degree revealed amino acid substitution at the DRDR of the *fop1* gene (Kai *et al.*, 1999; Matsuoka *et al.*, 2000, 2003, 2007; Williams *et al.*, 2000; Lee *et al.*, 2001; Maeda *et al.*, 2001; Cambau *et al.*, 2006). An isolate with mutation ACC to GCC at codon 53 was demonstrated to be resistant with low degree (Cambau *et al.*, 2006), though it is not clear whether dapsone resistance with low degree is true resistance (Matsuoka *et al.*, 2007). Other isolates with this mutation were found to be resistant to dapsone with intermediate degree. Therefore contradiction between mutation in the *folP1* gene and the results obtained by the mouse footpad drug susceptibility test has been encountered for only one case so far.

Although the direct sequencing of PCR products is definitive and allows rapid detection of resistant cases, it has the disadvantage of requiring expensive apparatus and high sequencing costs, so it is not practical in many developing countries. The heteroduplex method (HAD) (Williams *et al.*, 2001) and the PCR-single-strand conformation polymorphism method (SSCP) (Honoré *et al.*, 1993) have been applied to the detection of mutants to overcome these disadvantages. The HAD method can identify mutations in the PCR-amplified fragments by the electrophoretic mobility difference of heteroduplexes of wild-type products and test sample products, while the SSCP method analyses that of single-stranded products. However, neither the HAD nor the SSCP method fully meets the required conditions in developing countries, since these methods demand complicated procedures and both detect silent mutations as resistant mutations. The recently developed LineProbe assay based on reverse hybridization can detect rifampicin-resistant *M. leprae* simply and rapidly, but it cannot provide susceptibility information for other anti-leprosy drugs. The multiple-primer PCR amplification refractory mutation system is relatively simple but detects only nucleotide mutations and cannot distinguish silent mutations from missense mutations (Sapkota *et al.*, 2008)

Our present study aimed to exploit a rapid, simple and simultaneous drug susceptibility test for three key anti-leprosy drugs to solve defects of each method previously reported, based on DNA–DNA hybridization using a DNA microarray. The novel method, designated LDS-DA, allows the simultaneous identification of mutations in three genes, responsible for resistance to dapsone, rifampicin and the quinolones. Easy accessibility and high reproducibility demonstrated by the studies with clinical materials in two developing countries revealed the superior applicability of this method. Only five discordant results were found in 136 specimens examined. Three discordant results, two in Myanmar on *folP1* and one in the Philippines on *rpoB*, showed faint reactions on multiple spots probably caused by some technical errors. In the remaining two discordant results found in the Philippines, no signal was found at any position in row 55 of *folP1*. These samples were shown to carry a mutation at codon 55 in *folP1* from the wild-type CCC to CGT (Table 2), which was recently revealed to be associated with dapsone resistance (Cambau *et al.*, 2006) and was not covered by the oligonucleotide array on the LDS-DA. Although the signal was found neither at the wild-type nor at the mutant position in row 55 of *folP1*, this result can be taken as suggestive of dapsone resistance. The absence of a positive signal in the wild-type position implies the existence of base substitutions in the region covered by the oligonucleotide. Similar translation criteria have been applied to rifampicin-resistant *M. tuberculosis* by the commercially available INNO-LiPA Rif TB assay (Rossau *et al.*, 1997). Other possible mutation(s) related to drug resistance can also be distinguished under the same criteria.

The monitoring of drug-resistant leprosy cases has been recommended in order to maintain the effectiveness of chemotherapy for leprosy (Ji, 2002; Matsuoka *et al.*, 2007). The LDS-DA method developed in this study seems to be a simple and robust tool to assess the drug susceptibility of *M. leprae* in developing countries, where susceptibility testing is rarely applied. Comprehensive data on the prevalence of resistant cases shows that the level of drug resistance is low in some endemic countries (Matsuoka *et al.*, 2007). It is therefore recommended to apply this method to samples from intractable cases and relapsed cases, to examine the susceptibility to anti-leprosy drugs and ensure effective treatment. Additionally, the capacity of the LDS-DA method to identify the positions of mutations can be utilized for molecular epidemiological and geographical studies on the spread of drug-resistant *M. leprae.*