The total numbers of cells and macrophages in BAL fluid were significantly decreased, and the numbers of lymphocytes and eosinophils were significantly greater, after DEP exposure in Nrf2 $^{-/-}$ mice than in Nrf2$^{+/+}$ mice. It is interesting that the changes in these differential cell counts in C57BL/6 Nrf2 $^{-/-}$ mice 8 weeks after DEP exposure were similar to those previously reported in C57BL/6 wild-type mice 6 months after DEP exposure [14]. IL-12 influences the cytokine profile after T-cell activation [30]. Eosinophil recruitment into inflammatory sites is a complex process regulated by a number of cytokines including IL-13 [31]. Our findings suggest that IL-13 may be involved in eosinophil recruitment into the airways of Nrf2 $^{-/-}$ mice after DEP exposure, possibly indicating the presence of novel lymphocyte-directed chemokines [32,33]. We found that the level of TARC, but not that of eotaxin or RANTES, was significantly increased. Dias-Sanchez et al. have suggested that the effects of DEP on cytokine/chemokine expression are not global or non-specific [34]. Among these CC chemokines, TARC was the first one shown to selectively chemoattract T lymphocytes [32]. TARC was subsequently identified by induction of T-cell chemotaxis, especially in Th2-type CD4$^{+}$ T lymphocytes [33]. TARC is a pivotal chemokine for the development of Th2-dominated experimental asthma with eosinophilia and AHR [35]. Our present results indicate that TARC is also an important chemokine in the development of Th2-dominated oxidative stress-induced airway inflammation after DEP exposure.

Although it is reported that the effects of DEP-induced oxidative stress initiate and exacerbate airway allergic responses through enhanced IgE production [36,37], in the low-dose DEP exposure systems, no remarkable changes in IgE were evident in serum of Nrf2 $^{-/-}$ mice after DEP exposure.

In response to DEP exposure in the present study, there was increased induction of antioxidant enzyme mRNA in the lungs of Nrf2$^{+/+}$ mice. γ GCLm and GCLc are involved in glutathione (GSH) synthesis. GSH is the major intracellular thiol antioxidant that acts directly as a ROS scavenger. The GSH redox system plays a critical role in determining intracellular redox balance and antioxidant function [38]. GSR uses quinine oxidoreductase 1 (NADPH) for regeneration of reduced glutathione. G6PD, the enzyme involved in NADPH regeneration, was also considerably induced in Nrf2$^{+/+}$ mice in response to DEP. Our data clearly show that Nrf2 regulates several antioxidant enzyme genes that block oxidative stress and inflammation induced by DEP. The mRNA expression of other Nrf2-regulated antioxidant enzymes including GST-α3, GST-p2, GST-M1, SOD2 and HO-1 also increased in the Nrf2$^{+/+}$ mice. Our data indicate that Nrf2 deficiency results in reduced gene expression of antioxidant enzymes that block oxidative injury, leading to enhancement of inflammatory cell activity and AHR.

It has been reported that the pathogenesis of allergic asthma is related to oxidative stress [16]. In conjunction with allergens, DEP act as an adjuvant to enhance allergic responses such as expression of cytokines/chemokines and increased AHR [12,39]. It is conceivable that DEP exaggerate allergic asthmatic responses, and that the responses result from oxidative stress induced by DEP exposure.

In this study we showed for the first time that disruption of the Nrf2 gene facilitated susceptibility to airway inflammatory responses induced by inhalation of low-dose DEP in mice. These results strongly suggest that DEP-induced oxidative stress and host antioxidant responses play a key role in the development of DEP-induced airway inflammation, and may contribute to exaggeration of lung diseases related to oxidative stress such as allergic asthma.

## Acknowledgment

This study was supported in part by the Pollution-Related Health Damage Compensation and Prevention Association of Japan.

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# 16S rRNA遺伝子およびITS-1領域をターゲットとしたInvader法による23菌種の抗酸菌の同定

## —臨床分離株を用いたDDH法との比較検討—

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結核 Vol. 83 No.7 July 2008

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**要旨**：〔目的〕われわれは，16S rRNA遺伝子あるいは16S-23S rRNA遺伝子 internal transcribed spacer（ITS-1）領域の菌種特異的塩基配列にプローブを設定することにより23菌種の抗酸菌を同定できるInvader法を開発した。今回，多数の臨床分離株を用いInvader法とDNA-DNA hybridization（DDH）法の同定結果を比較検討した。〔方法〕2005年11月～12月の間にDDH法による菌種同定を受託した小川培地培養菌636株を評価に用いた。Invader法では13種類のプローブセットを用い，高温蛍光マイクロプレートリーダーで蛍光を計測することにより菌種を同定した。DDH法，アンプリコアPCR法，アキュプローブ法による測定はキットの添付文書に従った。同定不一致株については16S rRNA遺伝子の塩基配列を解析し菌種を決定した。〔結果〕今回調べた636株中615株（96.7%）はInvader法で同定可能であった。DDH法で同定対象になっていない*M. lentiflavum*が14株，*M. parascrofulaceum*が3株，*M. intermedium*が1株Invader法で同定された。一方，DDH法では，1回目の測定で636株中511株（80.3%），2回測定を繰り返すことにより580株（91.2%）の同定が可能であった。Invader法で複数菌種陽性となった8株を除いた628株のうち551株（87.7%）の結果はInvader法とDDH法で一致した。両法の結果の不一致例は，主に*M. gordonae*，*M. avium*，*M. lentiflavum*あるいは*M. intracellulare*で認められた。同定不一致株について，16S rRNA遺伝子のシークエンス解析などによりInvader法の結果が正しいことを確認した。〔結語〕臨床上重要な23菌種の抗酸菌の同定を目的としたInvader法により，DDH法に比べてより正確に抗酸菌種を分類および同定できることがわかった。また，臨床分離株のおよそ97%がこの方法で同定可能であった。

**キーワーズ**：抗酸菌の同定，Invader法，16S rRNA遺伝子，ITS-1領域，DDH法

## はじめに

非結核性抗酸菌による感染症は年々増加傾向にある[1,2]。現在，約110種類以上の抗酸菌菌種が知られており，それらは環境に広く分布している。その多くはヒトに対する病原性をもたないが，3分の1程度の菌種はヒトに対して病原性がある。*M. avium*と*M. intracellulare*はヒトの日和見感染症の起因菌であり，わが国では非結核性抗酸菌症の70%を占める。また，*M. kansasii*も非結核性抗酸菌症の起因菌であり全体の約20%である。非結核性抗酸菌症は，臨床症状や病理組織学的所見だけでは結核との鑑別が困難な場合があり，また菌種により薬剤感受性が異なることから，適切な診断や治療のためには迅速な菌種の同定が要求される[3]～[5]。

近年，遺伝子解析に基づいた様々な抗酸菌同定法が開発されている[6,7]。16S rRNA遺伝子[8,9]，16S-23S rRNA遺伝子internal transcribed spacer（ITS-1）領域[10]，*hsp65*（65 kDa heat shock protein）遺伝子[11]あるいは*rpoB*（DNA依存性RNAポリメラーゼβサブユニット）遺伝子[12]の塩基配列を解析し，相同性を調べる方法などが報告されて

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(Received 9 Jan. 2008 / Accepted 17 Mar. 2008)

いる。日本では，主に以下の2種類のキットが広く使用されている。アキュプローブ法は16S rRNAの可変領域をターゲットとし，菌種特異的なDNAプローブを用いて，RNA-DNAのハイブリダイゼーション法により同定を行う方法である[13)~15)]。同定可能菌種は，結核菌群（*M.tuberculosis* complex），*M.avium* complex（MAC），*M.kansasii*，*M.gordonae*である。全染色体DNAをターゲットとしたDNA-DNAハイブリダイゼーション法のDDHマイコバクテリア‘極東’（以下DDH法とする）は，マイクロプレートに固定した基準株DNAと被検菌DNAの全塩基配列の類似度を測定することにより菌種を同定する方法である[16)17)]。同定可能菌種は，結核菌群，*M.avium*，*M.intracellulare*，*M.kansasii*，*M.gordonae*，*M.chelonae*，*M.abscessus*，*M.fortuitum*，*M.scrofulaceum*，*M.marinum*，*M.simiae*，*M.szulgai*，*M.gastri*，*M.xenopi*，*M.nonchromogenicum*，*M.terrae*，*M.triviale*，*M.peregrinum*の計18菌種である。

われわれは，核酸を増幅することなく一塩基配列の違いを特異的に検出できるInvader法[18)19)]を抗酸菌同定に応用し，16S rRNA遺伝子とITS-1領域の菌種特異的配列を検出するInvader法による抗酸菌17菌種同定法を開発した[20)]。今回，検出プローブを追加し，同定可能菌種数を23菌種としたInvader法の特徴をより詳細に検討するために，DDH法との結果を比較検討した。

## 材料と方法

（1）使用菌株

2005年11月から12月までに株式会社ビー・エム・エル総合研究所にDDH法による同定依頼のあった，2%小川培地（極東製薬工業）に発育した臨床分離636株を対象とした。また，American Type Culture Collection（ATCC）の18菌種19株と，Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH（DSMZ）の1菌種1株も2%小川培地に発育させた後，検討に使用した。

（2）Invader法

Invader法に用いたDNAは，DDH法でフェノール抽出後の水層残渣200 $\mu l$からMagneSil（プロメガ）を使用して調製した。Invader法は以下の要領で行った。抽出したDNAを，384-wellプレートの所定のウェルに3 $\mu l$ずつ，1検体当たり26ウェル（13種×二重測定）に分注した後，ミネラルオイル（シグマ）を6 $\mu l$重層した。95℃で10分間処理しDNAを熱変性させた後，13種類のプローブ溶液それぞれ3 $\mu l$（Signal probe/Invader oligonucleotide/FRET probe溶液2.6 $\mu l$とCleavase酵素0.4 $\mu l$の混合溶液）を所定のウェルに分注した（Table 1）。高温蛍光マイクロプレートリーダー（FluoDia T70，大塚電子）にセット，64℃に保温し15分間隔で4時間蛍光を計測した。蛍光強度は，蛍光色素FAMは486 nm/530 nm（励起波長/蛍光波長）で，また蛍光色素Redmond REDは560 nm/620 nmで測定した。各検体につき，Broad-range bacteriaプローブの反応がプラトーに達した時間におけるGenus *Mycobacterium*あるいは菌種特異的プローブの蛍光強度からfold-over-zero（FOZ）[19)]の値を算出し，FOZ値が2以上の場合を陽性とした。なお，Broad-range bacteriaとGenus *Mycobacterium*のプローブのみ陽性であった場合を抗酸菌属細菌と，またBroad-range bacteriaプローブのみ陽性であった場合を抗酸菌属以外の細菌と判定した。

（3）DDH法

DDHマイコバクテリア‘極東’（極東製薬工業）による菌種の同定はキットの添付書類に従い行った。1回目の測定で判定基準を満たさなかった被検菌株について

**Table 1** Probe sets used for the Invader assay

| Set no. | FAM dye | Redmond RED dye |
|---|---|---|
| 1 | *M.tuberculosis* complex | None |
| 2 | *M.avium* | *M.kansasii*/*M.gastri* |
| 3 | *M.intracellulare* | *M.chelonae* (ITS-1) |
| 4 | *M.abscessus*/*M.chelonae* | Genus *Mycobacterium* |
| 5 | *M.scrofulaceum* | *M.gordonae* |
| 6 | *M.fortuitum* | *M.farcinogenes* complex[a] |
| 7 | *M.peregrinum*/*M.septicum* | *M.porcinum* complex[b] |
| 8 | *M.terrae* | *M.nonchromogenicum* group[c] |
| 9 | *M.xenopi* | *M.intermedium* |
| 10 | *M.simiae* (ITS-1) | *M.lentiflavum* |
| 11 | *M.triviale* | *M.szulgai* |
| 12 | *M.gastri* (ITS-1) | *M.marinum*/*M.ulcerance* |
| 13 | Broad-range bacteria | *M.scrofulaceum*/*M.parascrofulaceum* (ITS-1) |

[a] *M.farcinogenes*, *M.senegalense* and *M.houstonense*
[b] *M.porcinum*, *M.boenickei* and *M.neworleansense*
[c] *M.nonchoromogenicum*, *M.terrae*, *M.hiberniae* and *M.arupense*

は，再度測定を行い，2回目の測定においても判定基準を満たさなかったものを「同定不能」とした。

（4）アンプリコア™マイコバクテリウム アビウム，イントラセルラーおよびアキュプローブ マイコバクテリウム アビウム コンプレックス

アンプリコア PCR 法は，Invader 法で使用した DNA を用いて，アンプリコア™マイコバクテリウム アビウムおよびイントラセルラー（ロシュ・ダイアグノスティックス）の添付書類に従って測定した。また，アキュプローブ法は，小川培地菌株を用いて，アキュプローブ マイコバクテリウム アビウム コンプレックスキットおよびゴルドネ（ジェンプローブ）の添付書類に従って測定した。

（5）16S rRNA 遺伝子 Top500 シークエンス解析

Invader 法と DDH 法で結果が一致しなかった株のうち，アンプリコア PCR 法あるいはアキュプローブ法で確認できない菌種について，16S rRNA 遺伝子約 1,500 塩基のうちの 5' 側およそ 500 塩基（Top500）の配列を解析した。Invader 法で使用した DNA 溶液 1.0 $\mu l$ を用いて以下の条件で PCR 反応を行った。PCR 反応には，10 × Buffer 5 $\mu l$，8 mM dNTP 1 $\mu l$，プライマー 2 $\mu l$，滅菌蒸留水 40.5 $\mu l$，AmpliTaq Gold（アプライドバイオシステムズ）0.5 $\mu l$ を混和し反応溶液とした。プライマーは，Forward：5'-TGGAGAGTTTGATCCTGGCTCAG-3' と Reverse：5'-TACCGCGGCTGCTGGCAC-3' をそれぞれ 0.4 nmol 使用した。反応は，GeneAmp PCR System 9700 サーマルサイクラー（アプライドバイオシステムズ）を用い，95℃ 8 分間の後，94℃ 30 秒，60℃ 30 秒，72℃ 45 秒を 35 サイクル行うことによって，16S rRNA 遺伝子の 5' 側のおよそ 500 塩基を増幅した。PCR 産物を 3% アガロース電気泳動で確認した後，AMPure Kit（アジェンコート）を用いて DNA の精製を行った。サイクルシークエンスは，Big Dye terminator v1.1 cycle sequencing kit（アプライドバイオシステムズ）を用いて行った。CleanSEQ Kit（アジェンコート）を用いて精製した後，3130 ジェネティックアナライザー（アプライドバイオシステムズ）より得られた塩基配列データを Ribosomal Differentiation of Microorganisms[21]（RIDOM; http://www.ridom-rdna.de/）にて相同性を調べ，基準株と 98.5% 以上一致した菌種で相同性最上位をその菌種として同定した[22]。なお，RIDOM データベースで検索した結果，基準株と 98.5% 以上一致する菌種が存在しなかった株，あるいは RIDOM データベースに登録されていない菌種が疑われた株については，Genbank（BLAST; http//www.ncbi.nlm.nih.gov/BLAST/）を用いて検索し，基準株と 98.5% 以上一致した菌種で相同性最上位をその菌種として同定した。

（6）*M. gordonae* の *rpoB* 遺伝子の PCR 制限酵素解析

Invader 法で *M. gordonae* と同定された菌株について，伊藤らの方法[23]に準じて *rpoB* の遺伝子型を決定した。PCR 増幅産物を制限酵素 *Hae*III で消化した後，3% アガロース電気泳動のフラグメントパターンにより A・B・C・D あるいはその他のクラスターに分類した。

## 結　果

（1）Invader 法と DDH 法の結果の比較

DDH 法では，1 回目の測定で 636 株中 511 株（80.3%）の同定が，また，2 回目の測定を実施することにより 580 株（91.2%）まで同定が可能であった。しかしながら，同定菌種と培地上の集落の性状あるいは着色が一致しない株も存在した。一方，Invader 法では 636 株中 615 株（96.7%）が同定可能であった。Invader 法で複数菌種陽性となった 8 株を除いた 628 株において，両同定法の結果の一致率は 87.7%（551/628）であった（Table 2）。

（2）Invader 法と DDH 法の同定結果の乖離について

Invader 法で *M. avium* あるいは *M. intracellulare* と同定された 400 株のうち，DDH 法で結果が一致しなったものが 15 株存在した（Table 2）。これらの株のアンプリコア PCR 法およびアキュプローブ法の結果は，Invader 法の結果とすべて一致した（Table 3）。

Invader 法で *M. gordonae* と同定された 68 株のうち，DDH 法で *M. gordonae* と同定されたのは 34 株（50.0%）のみであり，29 株は同定不能，5 株は *M. gordonae* 以外の菌種に同定された（Table 2）。これら 68 株の *rpoB* 遺伝子について RFLP 解析を行ったところ，クラスター A が 9 株，クラスター B が 12 株，クラスター C が 16 株，クラスター D が 26 株認められ，その他のパターンを示したものが 5 株（パターン E 4 株，パターン F 1 株）確認された（Table 4）。クラスター A の 9 株はすべて DDH 法で *M. gordonae* と同定されたが，他のクラスターあるいは他のパターンに分類された株の同定には共通性が認められなかった。一方，*M. gordonae* の標準菌株である ATCC14470$^T$ と ATCC35756 はともに DDH 法で *M. gordonae* と同定され，*rpoB* の遺伝子型はクラスター A であった。

*M. lentiflavum* は，636 株中 14 株（2.2%）に検出された（Table 2）。この 14 株のうち 7 株は，DDH 法で *M. simiae*，*M. intracellulare* あるいは *M. fortuitum* に同定された。なお，この 7 株はすべて，16S rRNA 遺伝子のシークエンス解析で *M. lentiflavum* と同定された（Table 3）。

DDH 法で *M. scrofulaceum* と同定された 5 株のうち 3 株は，Invader 法で *M. parascrofulaceum* と同定され，16S rRNA 遺伝子 Top500 の配列は，BLAST 検索により *M. parascrofulaceum* の基準菌株 ATCCBAA-614$^T$ の配列（Accession No. AY337273）と完全に一致した（Table 3）。

**Table 2** Comparison of the identification results obtained by Invader assay and DDH method

| *Mycobacteria* identified by Invader assay | Testing by DDH method | | |
|---|---|---|---|
| | No. of strains with agreed results | No. of strains with discrepant results | Identified as |
| *M. avium* | 266 | 13 | *M. intracellulare* (4), *M. terrae* (1), Unidentified (8) |
| *M. intracellulare* | 119 | 2 | *M. avium* (2) |
| *M. gordonae* | 34 | 34 | *M. gastri* (3), *M. szulgai* (1), *M. xenopi* (1), Unidentified (29) |
| *M. kansasii* | 51 | 0 | |
| *M. fortuitum* | 19 | 0 | |
| *M. lentiflavum* | 7[a] | 7 | *M. simiae* (3), *M. intracellulare* (3), *M. fortuitum* (1) |
| *M. abscessus* | 11 | 0 | |
| *M. tuberculosis* complex | 9 | 0 | |
| *M. chelonae* | 8 | 0 | |
| *M. nonchromogenicum* group | 3[b] | 5 | *M. terrae* (1), Unidentified (4) |
| *M. peregrinum*/*M. septicum* | 3[c] | 1 | *M. gordonae* (1) |
| *M. szulgai* | 3 | 0 | |
| *M. terrae* | 3 | 0 | |
| *M. parascrofulaceum* | 0 | 3 | *M. scrofulaceum* (3) |
| *M. scrofulaceum* | 2 | 0 | |
| *M. porcinum* complex | 0 | 2 | *M. fortuitum* (2) |
| *M. xenopi* | 1 | 0 | |
| *M. intermedium* | 1[a] | 0 | |
| *M. simiae* | 0 | 0 | |
| *M. marinum*/*M. ulcerance* | 0 | 0 | |
| *M. gastri* | 0 | 0 | |
| *M. triviale* | 0 | 0 | |
| *M. avium* and *M. intracellulare* | 0 | 5 | *M. avium* (2), *M. intracellulare* (2), *M. abscessus* (1) |
| *M. avium* and *M. gordonae* | 0 | 2 | *M. avium* (2) |
| *M. avium* and *M. chelonae* | 0 | 1 | *M. chelonae* (1) |
| *Mycobacterium* sp. | 7[a] | 8 | *M. intracellulare* (2), *M. gordonae* (1), *M. szulgai* (1), *M. terrae* (1), *M. scrofulaceum* (1), *M. gastri* (1), *M. triviale* (1) |
| Other bacteria | 4[a] | 2 | *M. intracellulare* (1), *M. xenopi* (1) |

[a]The result obtained by DDH method was unidentified.
[b]The result obtained by DDH method was *M. nonchromogenicum*.
[c]The result obtained by DDH method was *M. peregrinum*.

なお，RIDOMで検索した場合は，16S rRNA遺伝子の超可変B領域を含まずに解析するため，*M. simiae*（ATCC 15080株と100%，ATCC14470$^T$株と99.3%一致）と同定された。

*M. nonchromogenicum* groupは，636株中8株（1.3%）に検出された（Table 2）。DDH法で同定不能と判定された4株の16S rRNA遺伝子Top500の配列を調べた結果，No.187株はBLAST検索により*M. arupense*の基準株AR30097$^T$の配列（Accession No. DQ157760）と完全に一致し，No.626株は1塩基の違いであった（Table 3）。一方，No.619とNo.625菌株はともにRIDOMでは*M. terrae*のS281株（sqvIII）と完全に一致し，BLAST検索では*M. arupense*の基準株と4塩基異なるのが相同性上位であった。DDH法で，*M. terrae*と同定された菌株（No.318）もNo.619およびNo.625菌株と同じ配列を有していた。

Invader法で*M. porcinum* complexと同定された2株（No.511とNo.518）は，ともにDDH法で*M. fortuitum*と同定された（Table 2）。No.518株の16S rRNA遺伝子Top500の配列は，*M. porcinum*の基準菌株ATCC39693$^T$あるいは*M. neworleansense*の基準菌株ATCC49404$^T$の配列と100%一致しており，一方，No.511株は99.8%の一致であった（Table 3）。なお，No.511株はRIDOMでは，*M. fortuitum*のS358株（sqvIV）と100%一致していた。また，Invader法で*M. peregrinum*/*M. septicum*，DDH法で*M. gordonae*と同定されたNo.543株の16S rRNA遺伝子の配列は，*M. peregrinum*基準菌株ATCC700731$^T$あるいは*M. septicum*基準菌株ATCC14467$^T$の配列と100%一致していた。

Invader法では，636株中8株（1.3%）で複数菌種陽性となった（Table 2）。No.75の株を除き，DDH法では，Invader法で同定された2菌種のうちのいずれかの菌種で同定された。*M. avium*と*M. intracellulare*の両方が陽性となった5株はすべてアンプリコアPCR法で*M. avium*と*M. intracellulare*の両者が陽性であった（Table 3）。

（3）Invader法で抗酸菌属細菌あるいは抗酸菌属以外の細菌と分別された菌株

**Table 3** Discrepant results in identification of *Mycobacterium* species except *M.gordonae* between Invader assay and DDH method

| Strain | Invader assay | DDH method | Amplicor or AccuProbe[a,b] | 16S rRNA sequencing[a] (%) |
|---|---|---|---|---|
| 48 | *M.avium* | *M.intracellulare* | *M.avium* | NT |
| 230 | *M.avium* | *M.intracellulare* | *M.avium* | NT |
| 171 | *M.avium* | *M.intracellulare* | *M.avium* | NT |
| 217 | *M.avium* | *M.intracellulare* | *M.avium* | NT |
| 551 | *M.avium* | *M.terrae* | *M.avium* | NT |
| 15 | *M.avium* | Unidentified | MAC | NT |
| 145 | *M.avium* | Unidentified | MAC | NT |
| 282 | *M.avium* | Unidentified | MAC | NT |
| 324 | *M.avium* | Unidentified | MAC | NT |
| 363 | *M.avium* | Unidentified | MAC | NT |
| 476 | *M.avium* | Unidentified | MAC | NT |
| 481 | *M.avium* | Unidentified | MAC | NT |
| 489 | *M.avium* | Unidentified | MAC | NT |
| 567 | *M.intracellulare* | *M.avium* | *M.intracellulare* | NT |
| 614 | *M.intracellulare* | *M.avium* | *M.intracellulare* | NT |
| 111 | *M.lentiflavum* | *M.simiae* | NT | *M.lentiflavum* (100) |
| 345 | *M.lentiflavum* | *M.simiae* | NT | *M.lentiflavum* (100) |
| 135 | *M.lentiflavum* | *M.simiae* | NT | *M.lentiflavum* (99.8) |
| 70 | *M.lentiflavum* | *M.intracellulare* | Negative | *M.lentiflavum* (100) |
| 396 | *M.lentiflavum* | *M.intracellulare* | Negative | *M.lentiflavum* (99.8) |
| 544 | *M.lentiflavum* | *M.intracellulare* | Negative | *M.lentiflavum* (100) |
| 39 | *M.lentiflavum* | *M.fortuitum* | NT | *M.lentiflavum* (100) |
| 318 | *M.nonchromogenicum* group | *M.terrae* | NT | *M.arupense* (99.1) |
| 187 | *M.nonchromogenicum* group | Unidentified | NT | *M.arupense* (100) |
| 626 | *M.nonchromogenicum* group | Unidentified | NT | *M.arupense* (99.8) |
| 619 | *M.nonchromogenicum* group | Unidentified | NT | *M.arupense* (99.1) |
| 625 | *M.nonchromogenicum* group | Unidentified | NT | *M.arupense* (99.1) |
| 543 | *M.peregrinum*/*M.septicum* | *M.gordonae* | Negative | *M.peregrinum* (100), *M.septicum* (100) |
| 265 | *M.parascrofulaceum* | *M.scrofulaceum* | NT | *M.parascrofulaceum* (100) |
| 580 | *M.parascrofulaceum* | *M.scrofulaceum* | NT | *M.parascrofulaceum* (100) |
| 33 | *M.parascrofulaceum* | *M.scrofulaceum* | NT | *M.parascrofulaceum* (100) |
| 511 | *M.porcinum* complex | *M.fortuitum* | NT | *M.porcinum* (99.8), *M.neworleansense* (99.8) |
| 518 | *M.porcinum* complex | *M.fortuitum* | NT | *M.porcinum* (100), *M.neworleansense* (100) |
| 183 | *M.avium* and *M.intracellulare* | *M.avium* | *M.avium* and *M.intracellulare* | NT |
| 369 | *M.avium* and *M.intracellulare* | *M.avium* | *M.avium* and *M.intracellulare* | NT |
| 127 | *M.avium* and *M.intracellulare* | *M.intracellulare* | *M.avium* and *M.intracellulare* | NT |
| 581 | *M.avium* and *M.intracellulare* | *M.intracellulare* | *M.avium* and *M.intracellulare* | NT |
| 75 | *M.avium* and *M.intracellulare* | *M.abscessus* | *M.avium* and *M.intracellulare* | NT |
| 3 | *M.avium* and *M.gordonae* | *M.avium* | MAC and *M.gordonae* | NT |
| 449 | *M.avium* and *M.gordonae* | *M.avium* | MAC and *M.gordonae* | NT |
| 541 | *M.avium* and *M.chelonae* | *M.chelonae* | MAC | NT |

[a]NT: Not tested, [b]MAC: *Mycobacterium avium* complex

Invader法で抗酸菌属細菌あるいは抗酸菌属以外の細菌と分別された検体は，636株中21株（3.3%）であった（Table 5）。21株のうち4株は，菌種特異的プローブあるいはGenus *Mycobacterium*プローブがハイブリダイズする塩基領域内に1塩基の置換を有していた。なお，No.279株は，BLAST検索で*Mycobacterium*属IWGMT 90174株と100%一致したが，基準株との相同性が98.5%を超えるものはなく同定できなかった。一方，抗酸菌属以外の細菌と判定されたものが6株存在した。No. 559株は，DDH法で*M.intracellulare*，16S rRNA遺伝子

**Table 4** The results in identification of *M. gordonae* between Invader assay and DDH method

| Invader assay | DDH method | AccuProbe[a] | *rpoB*-RFLP | No. of strains |
|---|---|---|---|---|
| *M. gordonae* | *M. gordonae* | NT | A | 9 |
| *M. gordonae* | *M. gordonae* | NT | B | 4 |
| *M. gordonae* | *M. gordonae* | NT | C | 7 |
| *M. gordonae* | *M. gordonae* | NT | D | 13 |
| *M. gordonae* | *M. gordonae* | NT | Other (E) | 1 |
| *M. gordonae* | *M. xenopi* | *M. gordonae* | B | 1 |
| *M. gordonae* | Unidentified | *M. gordonae* | B | 7 |
| *M. gordonae* | *M. gastri* | *M. gordonae* | C | 1 |
| *M. gordonae* | Unidentified | *M. gordonae* | C | 8 |
| *M. gordonae* | *M. gastri* | *M. gordonae* | D | 2 |
| *M. gordonae* | Unidentified | *M. gordonae* | D | 11 |
| *M. gordonae* | Unidentified | *M. gordonae* | Other (E) | 3 |
| *M. gordonae* | *M. szulgai* | *M. gordonae* | Other (F) | 1 |

[a]NT: Not tested

**Table 5** The strains which were not identified by Invader assay (%)

| Strain | Invader assay | DDH | AccuProbe[a, b] | 16S rRNA sequencing[a] (%) |
|---|---|---|---|---|
| 121 | *Mycobacterium* sp. (Low signal for *M. gordonae*) | *M. gordonae* | *M. gordonae* | *M. gordonae* (99.3) |
| 630 | *Mycobacterium* sp. (Low signal for *M. gordonae*) | Unidentified | *M. gordonae* | *M. gordonae* (99.3) |
| 279 | *Mycobacterium* sp. (Low signal for *M. avium*) | *M. scrofulaceum* | MAC | Unidentified |
| 451 | *Mycobacterium* sp. (Only *M. terrae* probe) | Unidentified | NT | NT |
| 402 | *Mycobacterium* sp. | *M. intracellulare* | MAC | *M. intracellulare* (98.8) |
| 72 | *Mycobacterium* sp. | *M. gastri* | NT | *M. neoaurum* (100) |
| 118 | *Mycobacterium* sp. | *M. terrae* | NT | *M. neoaurum* (99.8) |
| 640 | *Mycobacterium* sp. | *M. szulgai* | NT | *M. neoaurum* (100) |
| 32 | *Mycobacterium* sp. | Unidentified | NT | *M. celatum* [ATCC51130] (99.8) |
| 378 | *Mycobacterium* sp. | Unidentified | NT | *M. celatum* [ATCC51130] (99.8) |
| 335 | *Mycobacterium* sp. | Unidentified | NT | *M. mucogenicum* (99.1) |
| 514 | *Mycobacterium* sp. | Unidentified | NT | *M. mageritense* (100) |
| 9 | *Mycobacterium* sp. | *M. intracellulare* | Negative | *M. chlorophenolicum* (99.8) |
| 553 | *Mycobacterium* sp. | *M. triviale* | NT | *M. fortuitum* complex[c] (99.3) |
| 588 | *Mycobacterium* sp. | Unidentified | NT | *M. asiaticum* (99.1) |
| 559 | Other bacteria (Low signal for Genus *Mycobacterium*) | *M. intracellulare* | MAC | *M. chimaera* (99.3) |
| 246 | Other bacteria | *M. xenopi* | NT | *M. heckeshornense* (100) |
| 331 | Other bacteria | Unidentified | NT | *Gordonia sputi* (100) |
| 450 | Other bacteria | Unidentified | NT | *Corynebacterium jeikeium* (99.8) |
| 507 | Other bacteria | Unidentified | NT | *Gordonia otitidis* (100) |
| 573 | Other bacteria | Unidentified | NT | *Tsukamulla tyrosinosolvens* (99.8) |

[a]NT: Not tested
[b]MAC: *Mycobacterium avium* complex
[c]*M. fortuitum* complex: *M. fortuitum*, *M. farcinogenes*, *M. senegalense*, *M. houstonense*, *M. porcinum*, *M. boenickei*, *M. neworleansense* and *M. mucogenicum*

Top500の配列はBLAST検索で*M. chimaera*の基準菌株ATCC44623[T]の配列と99.3%一致し，RIDOMでは，*M. intracellulare*のATCC35770株（sqvIII）と99.8%一致していた。

（4）DDH法による基準菌株の同定

臨床分離株を用いた検討から，DDH法で誤同定する可能性の考えられる菌種について，その基準菌株を用いてDDH法を実施した結果をTable 6に示した。

## 考　察

今回，われわれの開発した16S rRNA遺伝子あるいはITS-1の菌種特異的塩基配列の検出により同定を行うInvader法と，全染色体DNAの相対類似度に基づき同定を行うDDH法との結果を比較検討した。その結果は，

**Table 6** Testing of the type strains by DDH method

| Species | Strain | Invader assay | DDH method |
|---|---|---|---|
| *M. tuberculosis* H37Rv | ATCC27294 | *M. tuberculosis* complex | *M. tuberculosis* complex |
| *M. avium* subsp. *avium* | ATCC25291 | *M. avium* | *M. avium* |
| *M. intracellulare* | ATCC13950 | *M. intracellulare* | *M. intracellulare* |
| *M. fortuitum* | ATCC6841 | *M. fortuitum* | *M. fortuitum* |
| *M. scrofulaceum* | ATCC19981 | *M. scrofulaceum* | *M. scrofulaceum* |
| *M. szulgai* | ATCC35799 | *M. szulgai* | *M. szulgai* |
| *M. xenopi* | ATCC19250 | *M. xenopi* | *M. xenopi* |
| *M. simiae* | ATCC25275 | *M. simiae* | *M. simiae* |
| *M. lentiflavum* | ATCC51985 | *M. lentiflavum* | Unidentified |
| *M. parascrofulaceum* | ATCCBAA-644 | *M. parascrofulaceum* | *M. scrofulaceum* |
| *M. neoaurum* | ATCC25795 | *Mycobacterium* sp. | *M. scrofulaceum* |
| *M. heckeshornense* | DSM44428 | Other bacteria | *M. xenopi* |
| *M. chlorophenolicum* | ATCC49826 | *Mycobacterium* sp. | Unidentified |
| *M. farcinogenes* | ATCC35753 | *M. farcinogenes* complex | Unidentified |
| *M. senegalense* | ATCC35796 | *M. farcinogenes* complex | *M. fortuitum* |
| *M. houstonense* | ATCC49403 | *M. farcinogenes* complex | *M. fortuitum* |
| *M. porcinum* | ATCC33776 | *M. porcinum* complex | *M. fortuitum* |
| *M. neworleansense* | ATCC49404 | *M. porcinum* complex | Unidentified |

両測定法の原理が強く反映されたものであった。

Invader法は，プローブを設定した菌種特異的塩基配列に依存した反応であり，特にシグナルプローブが100%マッチした菌株DNAのみを検出するため，抗酸菌菌種同定において非常に高い特異性を有した方法であることが再確認された。DDH法で同定可能な18菌種および臨床分離株から比較的高頻度に分離される菌種の検出プローブを組み合わせて設定することにより，臨床分離株の約97%を同定することが可能であった。

DDH法では，同定対象になっていないが臨床検体から比較的高頻度に分離される *M. lentiflavum*, *M. parascrofulaceum*, *M. neoaurum* などの菌種や，'*M. gordonae*-like'[21]を他の菌種に同定してしまうケースが確認された。また，DDH法では，*M. marinum* と遺伝学的に近縁な *M. ulcerans* と *M. shinshuense* を *M. marinum* と判定してしまう報告[24]や *M. heckeshornense* を *M. xenopi* と判定してしまう報告がある。さらに，*M. scrofulaceum*, *M. nonchromogenicum* あるいは *M. fortuitum* などの菌種では生化学性状による同定法との相関が悪いことが知られている[25)~27)]。DDH法は，「基準株に対して染色体DNAの定量的DNA/DNAハイブリダイゼーション試験で70%以上の類似度があり，ハイブリッドの安定度（ΔTm）が5度以内に収まる菌株の集団を同一種とする定義[28]」に準拠した方法である。しかし，被検菌株が固相された菌種以外であってもこの判定基準を満たしてしまう例が散見されている。すなわち，DDH法の判定基準は相対類似度に基づいて18菌種を識別する基準となっているが，近年非結核性抗酸菌の新菌種の提案は急増しており，判定基準設定時に含まれなかった類縁菌種を同定する場合には特に注意が必要である。

われわれの開発したInvader法による抗酸菌同定法も改善の余地はある。例えば，*M. nonchromogenicum* と *M. arupense*，*M. marinum* と *M. ulcerance* および *M. shinshuense* を区別することができない点である。このような場合は，種内で保存された菌種特異的な遺伝子配列を検索し，そこにプローブを設定することで分類が可能となる。なお，*M. arupense* は，2006年に *M. nonchromogenicum* に近縁な臨床分離株から同定された菌種[29]で，正木らが新菌種として報告していた *M. kumamotonense*[30]と同一菌種である。一方，データベースに登録されていない16S rRNA遺伝子配列を有する臨床分離株も稀ではあるが存在し，Invader法で同定できないケースもあった。この場合，プローブを数種類ミックスすることにより同定が可能となり，実際，*M. gordonae* はシグナルプローブを追加することによりNo.121とNo.630の株の同定が可能となった。さらに，*Mycobacterium* sp. と判定された株の同定率を上げるために，16S rRNA遺伝子の超可変A領域にプローブを設定することにより，データには示していないが，*M. neoaurum*[31]，*M. celatum*[32]，*M. mucogenicum*[33)34)]，*M. mageritense*[35]および *M. heckeshornense*[36)37)]の同定が可能であることを確認している。

また，今回の検討では，さまざまな非結核性抗酸菌が同定された。*M. lentiflavum* は1996年にヒトの脊椎椎間板炎病巣から分離された菌種で，喀痰，胃液，尿などからも分離例が報告されている[38]。近年，分子遺伝学的同定法の進歩により，本邦での分離同定例も増加し，本検討でも臨床分離株の約2%が *M. lentiflavum* であった。また，*M. parascrofulaceum* は2004年にTurenneらによって

*M. scrofulaceum*や*M. simiae*として同定されていたATCC株の中から新菌種として提案された菌種であり[39,40]，*M. simiae*と相同性の高い16S rRNA遺伝子とITS-1を有するが，生化学性状は*M. scrofulaceum*に類似し，*M. scrofulaceum*には存在しない16S rRNA遺伝子の超可変B領域に存在する12塩基を有していることが特徴的である。この領域の12塩基配列は迅速発育菌と*M. simiae* complexの遅速発育菌での保有が確認されている。一方，ITS-1の領域は，*M. simiae*と*M. scrofulaceum*に非常に相同性の高い配列を有している。これまでに日本での*M. parascrofulaceum*の分離の報告はなく，本検討における分離例が初めてである。さらに，今回新たにプローブを設定した*M. farcinogenes*/*M. senegalense*/*M. houstonense*あるいは*M. porcinum*/*M. boenickei*/*M. neworleansense*の菌種と*M. peregrinum*と*M. septicum*などを加えたグループを「*M. fortuitum* complex」と称することがあるように，遺伝学的には非常に近縁な菌種である。*M. houstonense*，*M. boenickei*，*M. neworleansense*は，2004年に新菌種の提案がされる以前は，*M. fortuitum* third biovariant complexと分類されていた経緯がある[41]。

近年，MGIT培地などの液体培地の導入や塩基配列解析などの遺伝子解析による同定方法の開発により，これまであまり同定されなかった菌種や新菌種が報告されるようになってきた。菌種の同定に基づき治療内容の選択が行われることから，正確にかつ臨床上重要な多くの菌種の同定が可能な測定系が望まれている。われわれの開発したInvader法による抗酸菌同定法は，これらの要求を満たした同定方法であり，臨床に広く使用されることを期待する。

## 謝　辞

本検討を実施するにあたり，多くのご助言を頂きました広島県環境保健協会の斎藤肇先生に深く感謝致します。また，本報告にあたり株式会社ビー・エム・エルの江頭徹氏，霜島正浩氏および山本英俊氏のご協力に感謝致します。

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Original Article

# IDENTIFICATION OF 23 MYCOBACTERIAL SPECIES BY INVADER ASSAY WITH TARGETING 16S rRNA GENE AND ITS-1 REGION
## — Comparison with DDH Method in Clinical Isolates —

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**Abstract** [Purpose and Method] The Invader assay was developed to identify 23 mycobacterial species using probes derived from the species-specific region of the 16S rRNA gene and the 16S−23S rRNA internal transcribed spacer 1 (ITS-1) region, with minor modifications of our previous study. In the present study, we compared the identification capability between the Invader assay and DNA-DNA hybridization (DDH) method. DDH is commonly used to identify non-tuberculosis mycobacterium in Japan and 636 clinical mycobacterial strains cultured on Ogawa slants were tested.

[Results] The Invader assay could identify 615 (96.7%) of the 636 strains. The results contained 14 *M. lentiflavum*, 3 *M. parascrofulaceum* and 1 *M. intermedium*, which were undetectable with DDH method. On the other hand, DDH method could identify 580 (91.2%) strains with duplicate assay. Of 628 strains except 8 strains identified as a few species by Invader assay, 551 (87.7%) strains were identified as the same species by two methods. Discordant results were mainly recognized for the identification of *M. gordonae*, *M. avium*, *M. lentiflavum* and *M. intracellurare*. The results of other methods targeting 16S rRNA indicated correctness of the Invader assay.

[Conclusion] These results indicate that Invader assay could identify more correctly than DDH method and could identify about 97% of clinically important mycobacterium.

**Key words**: Identification of mycobacteria, Invader assay, 16S rRNA gene, ITS-1 region, DDH method

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INFECTION AND IMMUNITY, Apr. 2008, p. 1547–1557 Vol. 76, No. 4
0019-9567/08/$08.00+0 doi:10.1128/IAI.01269-07


# Critical Involvement of Pneumolysin in Production of Interleukin-1α and Caspase-1-Dependent Cytokines in Infection with *Streptococcus pneumoniae* In Vitro: a Novel Function of Pneumolysin in Caspase-1 Activation▽

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Received 15 September 2007/Returned for modification 7 November 2007/Accepted 8 January 2008

**Pneumolysin is a pore-forming cytolysin known as a major virulence determinant of *Streptococcus pneumoniae*. This protein toxin has also been shown to activate the Toll-like receptor 4 (TLR4) signaling pathway. In this study, a mutant *S. pneumoniae* strain deficient in pneumolysin (Δ*ply*) and a recombinant pneumolysin protein (rPLY) were constructed. Upon infection of macrophages in vitro, the ability to induce the production of interleukin-1α (IL-1α), IL-1β, and IL-18 was severely impaired in the Δ*ply* mutant, whereas there was no marked difference in the induction of tumor necrosis factor alpha (TNF-α) and IL-12p40 between the wild type and the Δ*ply* mutant of *S. pneumoniae*. When macrophages were stimulated with rPLY, the production of IL-1α, IL-1β, and IL-18 was strongly induced in a TLR4-dependent manner, whereas lipopolysaccharide, a canonical TLR4 agonist, hardly induced these cytokines. In contrast, lipopolysaccharide was more potent than rPLY in inducing the production of TNF-α, IL-6, and IL-12p40, the cytokines requiring no caspase activation. Activation of caspase-1 was observed in macrophages stimulated with rPLY but not in those stimulated with lipopolysaccharide, and the level of activation was higher in macrophages infected with wild-type *S. pneumoniae* than in those infected with the Δ*ply* mutant. These results clearly indicate that pneumolysin plays a key role in the host response to *S. pneumoniae*, particularly in the induction of caspase-1-dependent cytokines.**

*Streptococcus pneumoniae* is a gram-positive bacterium that causes bacterial pneumonia, otitis media, bacterial meningitis, and septicemia (32). Due to the severe disease burden and mortality, the emergence of drug-resistant clinical isolates (14, 36), the lack of a universally effective vaccine (17, 39), and an increase in the number of immunocompromised patients, it is increasingly important to understand the pathogenic processes of pneumococcal disease in order to develop novel therapeutic modalities and an effective vaccine.

Pneumolysin (PLY), a 53-kDa protein toxin produced by virtually all clinical isolates of *S. pneumoniae*, has been regarded as a key virulence factor of this bacterium (7) and as one of the candidates for vaccine development against pneumococcal infection (21). PLY is one of the cholesterol-dependent cytolysins (24) that are known to form ring- or arc-shaped pores on cholesterol-containing membranes and whose activity is blocked by free cholesterol (40, 50). PLY is a multifunctional protein toxin that causes cytolysis and induces complement activation and the production of cytokines and nitric oxide (5, 9, 18, 33, 43). However, the mechanisms by which PLY affects the host defense are only partly understood.

In mammals, the sensing of microbial components by innate immune cells, such as macrophages and dendritic cells, is initiated by the recognition of conserved and unique pathogen-derived structures via pattern recognition molecules, such as Toll-like receptors (TLRs) (31). TLRs, transmembrane proteins belonging to the Toll/interleukin-1 (Toll/IL-1) receptor family (16), mediate host immune responses by inducing proinflammatory cytokines and costimulatory molecules through the activation of nuclear factor kappa B (NF-κB) and mitogen-activated protein kinases (MAPKs) after recognition of specific agonists (19). Among several TLRs, TLR4 plays a critical role in the recognition of lipopolysaccharide (LPS), a major component of the outer membranes of gram-negative bacteria, in cooperation with LPS-binding protein, CD14, and MD2 (1, 25, 51, 53), and plays a critical role in the host defense against gram-negative pathogens (8, 37). Several recent studies have shown that the association of TLR4 with PLY (and other cholesterol-dependent cytolysins) initiates an intracellular signaling cascade resulting in the activation of NF-κB (20, 27, 41, 49, 51). For pneumococcal infection, Malley et al. (27) reported an important role of TLR4 in the host defense with the finding that C3H/HeJ mice, which display mutant nonfunctional TLR4, were more susceptible than wild-type (wt) mice to invasive disease after pneumococcal colonization in the nasopharynx. Moreover, a protective role of TLR4 in the pneumococcal pneumonia model was also reported (8). Thus, it appears that PLY, a TLR4 agonist in this gram-positive pathogen (27), is involved in the induction of a host protective response against *S. pneumoniae*.

Several reports have shown that proinflammatory cytokines, such as IL-1, tumor necrosis factor alpha (TNF-α), IL-6, and IL-18, play protective roles against pneumococcal infection (22, 26, 48, 54, 55). Moreover, gamma interferon has been

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▽ Published ahead of print on 14 January 2008.

demonstrated to protect mice against infection with *S. pneumoniae* by promoting the accumulation of neutrophils in the infected lung (34). In addition, exogenous administration of IL-12 improved the innate defense against *S. pneumoniae* in the lung by inducing gamma interferon production (23). However, the involvement of PLY in the production of various cytokines induced in pneumococcal infections has yet to be clarified.

In the present study, we constructed an in-frame deletion mutant with a mutation in the *S. pneumoniae ply* gene and a recombinant protein of PLY to analyze the precise role for PLY in the host cytokine response to *S. pneumoniae*. Using an in vitro model of infection, we compared the levels of various proinflammatory cytokines secreted from macrophages, the cells comprising the front line of host defense and the innate immune response, after infection with the wt or *ply*-deficient mutant strain of *S. pneumoniae*. This study revealed a unique function of PLY in the induction of caspase-1-dependent cytokine production that could not be observed with LPS, a canonical TLR4 ligand.

TABLE 1. Oligonucleotide primers used in this study

| Primer | Nucleotide sequence (5′–3′) |
|---|---|
| IL-1α-F | CTCTAGAGCACCATGCTACAGAC |
| IL-1α-R | TGGAATCCAG-GGGAAACACTG |
| IL-1β-F | AAGCTCTCCACCTCAATGGACAG |
| IL-1β-R | CTCAAACTCCAC-TTTGCTCTTGA |
| IL-18-F | ACTGTACAACCGCAGTAATACGG |
| IL-18-R | AGTGAACATTACAGATTTATCCC |
| TNF-α-F | GGCAGGTCTACTTTGGAGTCATTGC |
| TNF-α-R | ACATTCGAGGCTCCAGTGAATTCCA |
| IL-6-F | GAGGATACCACTCCCAACAGACC |
| IL-6-R | AAGTGCATCATCGTTGTTCATACA |
| IL-12p40-F | TCCGGAGTAATTTGGTGCTTCACA |
| IL-12p40-R | GCAAGAGACACAGTCCTGGG |
| GAPDH-F | TGCCCAGAACATCATCCCTG |
| GAPDH-R | AACACGGAAGGCCATGCCAG |
| PLY-F | CGATGGATCCTATGGCAAATAAAGCAGTAA |
| PLY-R | ACGCGGTACCCTAGTCATTTTTCTACCTTAT |
| P1 | ACACGGATCCTCAACAGGCACTCATCCACA |
| P2 | GCGCAAGCTTGGAGAATGCTTGCGAC AAAA |
| P3 | GCGCAAGCTTAAATCAGCCGTGGTTGGACT |
| P4 | ACACGGATCCCGCAAAGCCCTTTTTCTAGC |

## MATERIALS AND METHODS

**Experimental animals.** wt female C57BL/6 mice were purchased from Japan SLC (Shizuoka, Japan). C57BL/6 background TLR4 gene knockout (TLR4 KO) mice were purchased from Oriental Bioservices (Kyoto, Japan) and maintained in a specific-pathogen-free environment for use at 7 to 8 weeks of age. All animal experiments were approved by the Animal Ethics and Research Committee of Kyoto University Graduate School of Medicine.

**Bacterial strains and growth conditions.** A serotype 2 strain of *Streptococcus pneumoniae* D39 was purchased from the National Collection of Type Cultures (NCTC 7466; Central Public Health Laboratory, London, United Kingdom). *S. pneumoniae* was grown on tryptic soy agar (Difco Laboratories, Detroit, MI) with 5% (vol/vol) defibrinated sheep blood (Nacalai Tesque, Kyoto, Japan) and in Todd-Hewitt broth (Difco) supplemented with 0.5% yeast extract (THY) at 37°C and 5% $CO_2$ and subsequently stored at −80°C in THY plus 10% glycerol. For the preparation of bacterial stocks for macrophage stimulation, pneumococci were grown overnight on blood agar plates at 37°C and 5% $CO_2$. Colonies were inoculated into the THY medium, grown until mid-logarithmic phase (optical density at 600 nm [$OD_{600}$] = 0.5), and centrifuged at 6,000 × *g* for 15 min. The bacterial pellet was suspended in phosphate-buffered saline (PBS) and stocked at −80°C. The concentration was determined by viable cell counting on blood agar plates.

**Construction of *ply* deletion mutant.** A deletion mutant of *S. pneumoniae* D39 for the PLY gene (*ply*) was constructed by using homologous recombination-based allelic exchange. To generate the *ply* deletion, the upstream (733 bp) and downstream (692 bp) flanking regions of *ply* were PCR amplified from D39 genomic DNA, using the primer sets P1/P2 and P3/P4, respectively (primer sequences are given in Table 1). Primers P1 and P4 carried one BamHI site, and P2 and P3 carried HindIII sites in their 5′ ends. Amplified fragments were digested with HindIII and ligated. The resulting fusion gene product was amplified by PCR using primers P1 and P4, digested with BamHI, and then ligated with BamHI-digested vector DNA (pTN-E18EM) ($Amp^r$ $Em^r$). Plasmid pTN-E18EM is a pUC18-derived vector carrying ampicillin and erythromycin resistance genes and the multiple cloning site of pUC18. The erythromycin resistance gene (*emrC*) was amplified from plasmid pE194 by PCR and inserted into pUC18.

**Transformation into *S. pneumoniae* and selection of *ply* deletion mutant.** To carry out the transformation of the recombinant plasmid, frozen stocks of *S. pneumoniae* were thawed and diluted 1:20 in competence medium (tryptic soy broth [Difco], pH 8.0, 10% glycerol, 0.16% bovine serum albumin, 0.01% $CaCl_2$) containing competence-stimulating peptide 1 (100 ng/ml; Invitrogen, Carlsbad, CA). *S. pneumoniae* D39 was preincubated for 20 min at 37°C and 5% $CO_2$ and then incubated for 1 h with approximately 1 μg of DNA. The cells were plated on blood agar containing erythromycin, and transformants were obtained. For the selection of the *ply* deletion mutant, transformants were grown in THY medium without antibiotics and plated on blood agar without antibiotics, and then colonies were plated on replica plates with or without erythromycin. Erythromycin-sensitive colonies were selected, and *ply*-negative mutants were confirmed by PCR to have an absence of *ply*, using primers PLY-F and PLY-R (Table 1), and the presence of the upstream and downstream sequences of the *ply* gene. The absence of PLY in the *ply* deletion mutant was verified by Western blotting using a monoclonal antibody against PLY (NovoCastra Laboratories Ltd., Newcastle upon Tyne, United Kingdom).

**Production and purification of rPLY.** Full-length recombinant PLY (rPLY) was prepared as described previously (4). Briefly, the *ply* gene was cloned into the pQE-31 vector (Qiagen, Hilden, Germany), and the recombinant vector was transformed into *Escherichia coli* SG13009 (Qiagen) harboring a pREP4 plasmid, which contains *lacI* and kanamycin resistance genes. rPLY was produced in *E. coli* cells as a six-His-tagged protein by incubation of the transformants with 2 mM isopropyl-β-D-thiogalactopyranoside (Nacalai Tesque) at 25°C for 6 h. The *E. coli* cells were then harvested by centrifugation, incubated with lysis buffer (50 mM $NaH_2PO_4$, 300 mM NaCl, 20 mM imidazole, 1 mg/ml lysozyme, 200 U DNase I, pH 8.0), and disrupted by vortexing with 0.1-mm zirconia-silica beads (Bio-Spec Products, Inc., Bartlesville, OK). rPLY was then purified from the soluble fraction by use of a nickel-nitrilotriacetic acid column (Qiagen) under native conditions according to the manufacturer's instructions. Contaminating LPS was extensively removed using a Detoxi-Gel endotoxin-removing gel (Pierce Chemical Co., Rockford, IL). The level of LPS in the rPLY preparation was determined by the *Limulus* Color KY test (Wako Pure Chemical Industries, Osaka, Japan) and was found to be <0.4 pg/ml when the preparation was suspended in PBS at a protein concentration of 1 μg/ml. The purity was analyzed by Coomassie brilliant blue staining and immunoblotting using an anti-His-tag monoclonal antibody (penta-His antibody; Qiagen) after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

To inhibit its cytolytic activity, rPLY was treated with 20 μg/ml of cholesterol for 30 min on ice (35). Heat-treated PLY and LPS were prepared by heating the aqueous stock suspensions, in 50% glycerol-water (vol/vol) for PLY and in PBS for LPS, in a boiling water bath for 60 min (27).

**Isolation and stimulation of peritoneal macrophages.** Peritoneal exudate cells were collected from C57BL/6 wt and TLR4 KO mice 3 days after an intraperitoneal injection of 4% thioglycolate medium (Eiken Chemical, Tokyo, Japan). Cells were cultured in 48-well plates ($2 \times 10^5$ cells per well) in medium consisting of RPMI 1640 (Gibco-BRL Life Technologies, Rockville, MD) supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum (Gibco) for 2 h at 37°C and 5% $CO_2$. After removal of nonadherent cells, the adherent cells were used as peritoneal macrophages and infected with wt *S. pneumoniae* and its isogenic *ply* deletion mutant (Δ*ply*) at a multiplicity of infection (MOI) of 10 in gentamicin-free medium. To inhibit the growth of bacteria in the medium, 100 μg/ml of gentamicin (Gibco) was added to the cultures 8 h after infection, when the largest number of bacteria were associated with cells, and the cells were incubated for an additional 16 h. Supernatants were collected and stored at −80°C until they were assayed for cytokines. Similarly, peritoneal macrophages were stimulated with rPLY, LPS (from *E. coli* O55:B5; Sigma-Aldrich, St. Louis, MO), or $Pam_3CSK_4$ (Invivogen, San Diego, CA). In some experiments, polymyxin B

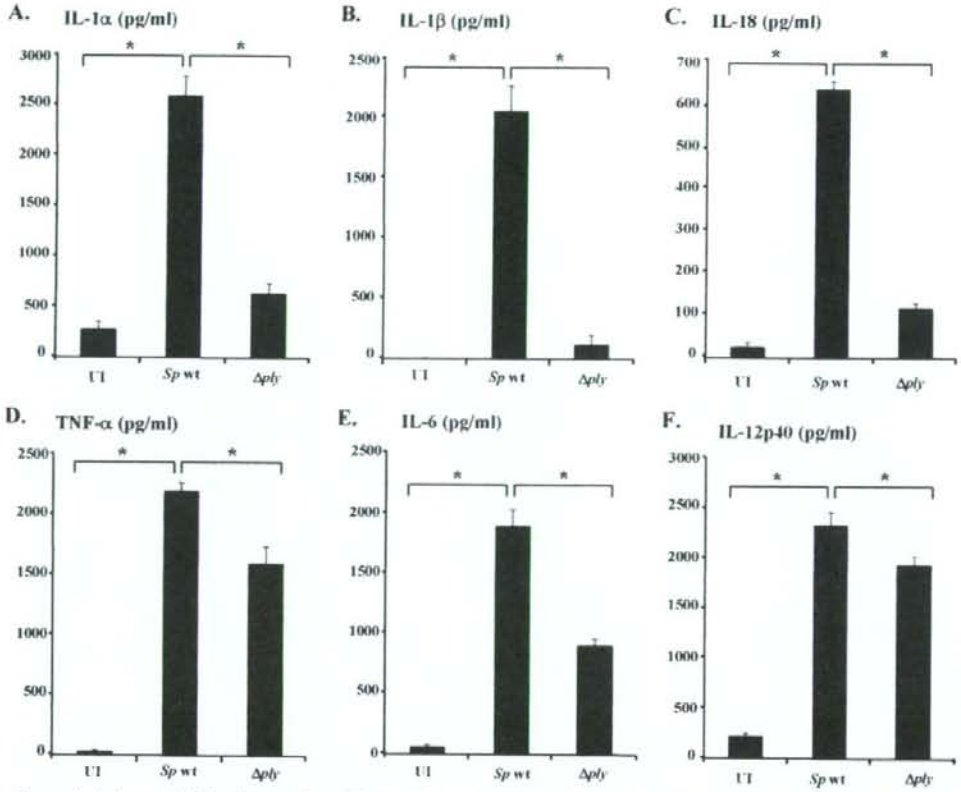


FIG. 1. Pneumolysin is essential for the secretion of IL-1α, IL-1β, and IL-18 in response to *Streptococcus pneumoniae*. Peritoneal macrophages were left uninfected (UI) or infected with wt *S. pneumoniae* (*Sp* wt) or the *ply* mutant (Δ*ply*) at a macrophage/bacterium ratio of 1:10 for 8 h. Cells were cultured for an additional 16 h in the presence of gentamicin (100 μg/ml), and culture supernatants were then collected. The amounts of IL-1α (A), IL-1β (B), IL-18 (C), TNF-α (D), IL-6 (E), and IL-12p40 (F) were determined using ELISAs specific for each cytokine. The results are representative of three similar experiments. The data are the means ± standard deviations for three determinations. *, $P < 0.05$ for uninfected cells compared to wt *S. pneumoniae*-infected cells and for wt *S. pneumoniae*-infected cells compared to Δ*ply* mutant-infected cells.

(PMB; Nacalai Tesque), z-VAD-fmk (Peptide Institute, Osaka, Japan), and z-YVAD-cmk (R&D Systems, Minneapolis, MN) were added to the cultures 30 min before stimulation with rPLY or LPS.

**Reverse transcription-PCR.** Total cellular RNA was extracted from peritoneal macrophages by using Nucleospin RNA II (Macherey-Nagel, Düren, Germany). Total RNA (0.2 μg) was treated with RNase-free DNase (Promega, Madison, WI) to eliminate contaminating DNA and then subjected to reverse transcription using random primers (Invitrogen) and ReverTra Ace (Toyobo, Osaka, Japan). PCR was performed using KOD-Plus DNA polymerase (Toyobo) under the following PCR conditions: 94°C for 15 s, 60°C for 30 s, and 68°C for 30 s. The reaction was extended by incubation at 68°C for 7 min. The samples were amplified for 28 to 30 cycles. The most appropriate number of amplification cycles for each cytokine was determined by preliminary experiments. PCR products were analyzed in 2% agarose gels. Primer sequences used for the amplification of specific genes by reverse transcription-PCR are shown in Table 1.

**Cytokine measurement.** Levels of secreted cytokines in culture medium were determined by two-site sandwich enzyme-linked immunosorbent assay (ELISA). ELISA kits for IL-1α and IL-1β were purchased from BD Biosciences (San Diego, CA). IL-6, TNF-α, and IL-12p40 kits were obtained from eBioscience (San Diego, CA), and an IL-18 kit was obtained from MBL (Nagoya, Japan). All samples were assayed according to the respective manufacturer's instructions.

**Western blot analysis.** For Western blotting, cells were lysed in 2× SDS-PAGE sample buffer (62.5 mM Tris-HCl, 2% SDS, 10% glycerol, 50 mM dithiothreitol, 0.1% bromophenol blue) and subjected to brief ultrasonication and boiling for 5 min. The lysates were resolved by SDS-PAGE and then transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA). Antibodies specific for mouse IκBα, p38 MAPK, phospho-p38 MAPK (Thr180/Tyr182), and phospho-ERK1/ERK2 (Thr202/Tyr204) were purchased from Cell Signaling Technology (Danvers, MA) and were used as recommended by the manufacturer.

**Caspase-1 activation assay.** To determine the activation of caspase-1, peritoneal exudate cells were seeded in 24-well tissue culture plates at $5 \times 10^5$ cells per well. After removal of nonadherent cells, the adherent cells were incubated with 30 μM biotinylated YVAD-cmk (Alexis Biochemicals, San Diego, CA) for 1 h. The cells were then either left unstimulated or stimulated with rPLY or LPS for 3 h at different concentrations. In the case of LPS plus ATP stimulation, cells were primed with LPS for 2.5 h and subsequently stimulated with 1 mM ATP (Amersham) for 30 min. Similarly, peritoneal macrophages were infected with wt *S. pneumoniae* and the Δ*ply* mutant at an MOI of 10 for 3 or 6 h. Cells were then washed with PBS three times and lysed with 1 ml lysis buffer consisting of 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 10% glycerol, 1% Triton X-100, 5 μg/ml leupeptin, 5 μg/ml aprotinin, 5 μg/ml pepstatin, and 1 mM phenylmethylsulfonyl fluoride. Lysates were centrifuged at 12,000 ×*g* for 1 min to remove cell debris. To adjust the amount of lysate recovered from each well, 10 μl of cleared lysate was subjected to Western blotting using a specific antibody for β-actin (Sigma), and the rest of the lysate was used for pull-down assay for detecting the active form of caspase-1. Activated caspase-1 (bound to biotinylated YVAD-cmk) was

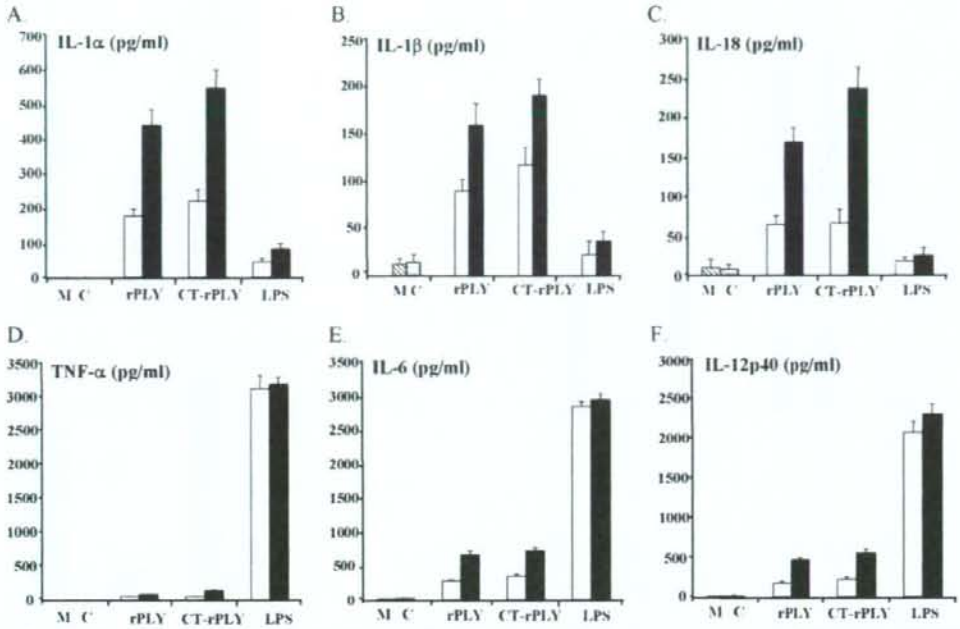


FIG. 2. Pneumolysin is a strong inducer of IL-1α, IL-1β, and IL-18 production. Peritoneal macrophages were left unstimulated (M), treated with cholesterol only (C), or stimulated with rPLY, cholesterol-treated rPLY (CT-rPLY), or LPS at a concentration of 0.1 μg/ml (white bars) or 1 μg/ml (black bars) for 24 h, and culture supernatants were then collected. The levels of cytokines in supernatants were determined by ELISA. The results are representative of three similar experiments. The data are the means ± standard deviations for three determinations.

concentrated with tetrameric avidin resin (Promega) and detected by Western blotting using anti-caspase-1 polyclonal antibody (MBL).

**Statistical analysis.** For comparisons between two groups, the Mann-Whitney U test was used, and statistical significance was determined as a $P$ value of $<0.05$. Multigroup comparisons of mean values were conducted by the Kruskal-Wallis test and the Games-Howell post hoc test ($P < 0.05$) after the confirmation of homogeneity of variances among the groups by using Bartlett's test.

## RESULTS

**Role of PLY in cytokine production by macrophages infected with *S. pneumoniae* in vitro.** To evaluate the involvement of PLY in inducing proinflammatory cytokines in response to *S. pneumoniae*, peritoneal macrophages from C57BL/6 mice were infected with live wt *S. pneumoniae* or the Δ*ply* mutant, and the levels of cytokines in supernatants were assayed. wt *S. pneumoniae* potently induced IL-1α, IL-1β, and IL-18, whereas the Δ*ply* mutant was unable to induce these cytokines, indicating a critical role of PLY in inducing IL-1α, IL-1β, and IL-18. In contrast, no marked difference was observed between the two strains in the ability to induce TNF-α, IL-6, and IL-12p40, suggesting a minor role of PLY in induction of these cytokines (Fig. 1). The impaired ability of the Δ*ply* mutant to induce cytokine production was not due to any enhanced cytotoxicity, as the release of lactate dehydrogenase from macrophages infected with the mutant never exceeded the level induced by wt *S. pneumoniae* (data not shown).

**PLY strongly induces IL-1α, IL-1β, and IL-18.** To confirm whether the *ply* gene product was responsible for the difference in cytokine induction between the *ply*-positive wt and *ply*-deficient mutant strains, we next examined the profiles of various cytokines induced by rPLY stimulation. Although it was difficult to determine the exact amount of PLY released from wt *S. pneumoniae* in our in vitro infection system, we tried to estimate the amount based on a previous report on the relationship between bacterial number and the amount of PLY protein (18). The amount of PLY released from the infection dose of wt *S. pneumoniae* used in this study was calculated to be 0.04 μg/ml. In a study by Malley et al. (27), rPLY was used at concentrations of 0.1 μg/ml to 10 μg/ml to determine TLR4-dependent cytokine-inducing activity. Taking these findings into consideration, we used rPLY in the present study at cytolytic (1 μg/ml) and sublytic (0.1 μg/ml) concentrations, which caused 53.0% and 5.1% release of lactate dehydrogenase from macrophages, respectively. Both doses of rPLY exhibited a stronger activity to induce IL-1α, IL-1β, and IL-18 than did LPS (Fig. 2) and TLR2 ligands, such as $Pam_3CSK_4$ (data not shown). In contrast, the ability of rPLY to induce IL-6, IL-12p40, and TNF-α was significantly lower than that of LPS. A treatment of 1 μg/ml or 0.1 μg/ml of rPLY with cholesterol resulted in the reduction of the cytolytic activity determined by lactate dehydrogenase release assay, by 95.3% and 100%, respectively. However, the cytokine-inducing activity was not affected by cholesterol pretreatment, even after the abolishment of cytolytic activity. Both cholesterol-treated rPLY and untreated rPLY induced similar levels of all cytokines tested (Fig.

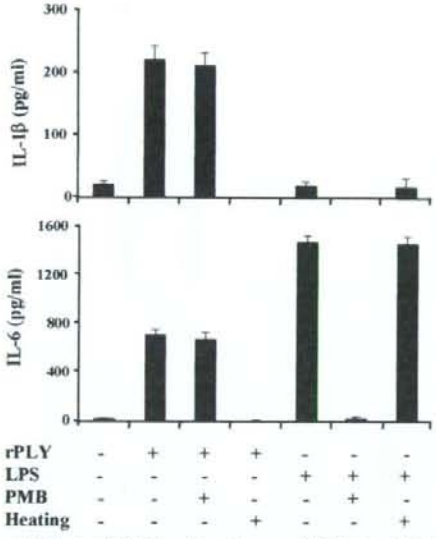


FIG. 3. Effects of PMB and heating on rPLY-induced IL-1β and IL-6 production. Peritoneal macrophages were stimulated with cholesterol-treated rPLY or LPS for 24 h, and the culture supernatant was then collected. The amounts of IL-1β (A) and IL-6 (B) in the culture supernatant were measured by ELISA. The effects of the addition of 0.5 μg of PMB/ml and heating at 100°C for 1 h were examined. The results are representative of three similar experiments. The data are the means ± standard deviations for three determinations.

2), indicating that cytokine-inducing activity and cytolytic activity of PLY may be dissociated. The absence of any stimulating or toxic activity was confirmed in the medium containing cholesterol alone.

To eliminate the possibility that cytokine induction by rPLY was due to contamination of LPS from *E. coli*, we added PMB to the cell culture before stimulation with rPLY and LPS. PMB did not affect rPLY-induced production of IL-1β or IL-6 (Fig. 3) as well as IL-1α, IL-18, TNF-α, and IL-12p40 (data not shown), whereas the same amount of PMB completely abolished the production of cytokines induced by LPS. Furthermore, when rPLY was heated at 100°C for 60 min, the ability to induce these cytokines was completely abrogated. Based on these findings, the possibility of contaminating LPS-induced cytokine production was ruled out.

**Supplementation with rPLY enhanced IL-1α, IL-1β, and IL-18 production by macrophages infected with the Δ*ply* mutant.** Although rPLY demonstrated a significantly higher activity in inducing IL-1α, IL-1β, and IL-18 than did LPS (Fig. 2), the levels were significantly lower than those induced by infection of macrophages with wt *S. pneumoniae* (Fig. 1). To examine whether high levels of these cytokines induced upon infection with wt *S. pneumoniae* were due to simultaneous stimulation with PLY and other bacterial components, we infected macrophages with the Δ*ply* mutant in the presence of rPLY. Costimulation with rPLY and the Δ*ply* mutant induced a greatly enhanced production of IL-1α, IL-1β, and IL-18, which was comparable to that induced by wt *S. pneumoniae* (Fig. 4). In the absence of rPLY, an increase in the dose of the

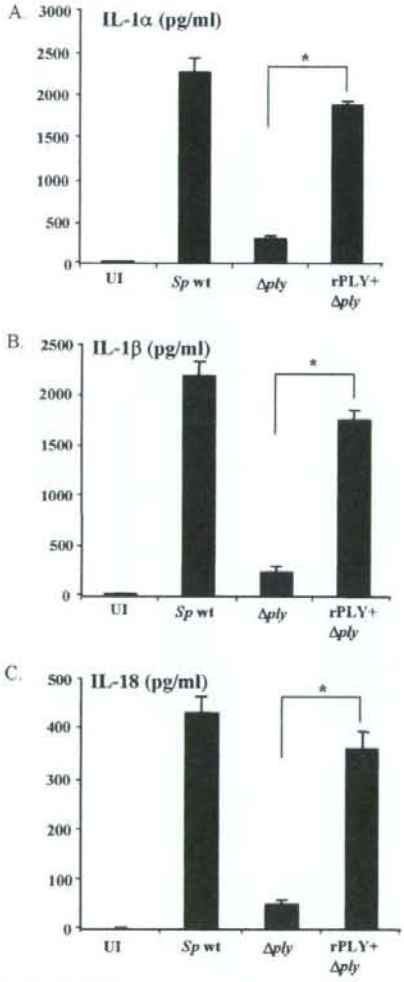


FIG. 4. Costimulation of rPLY with the Δ*ply* mutant significantly increased IL-1α, IL-1β, and IL-18 production by the Δ*ply* mutant. Peritoneal macrophages were left uninfected (UI) or infected with wt *S. pneumoniae* (*Sp* wt), the Δ*ply* mutant, or rPLY (1 μg/ml) plus the Δ*ply* mutant at a macrophage/bacterium ratio of 1:10. The amounts of IL-1α (A), IL-1β (B), and IL-18 (C) in culture supernatants were determined by ELISAs specific for each cytokine. The results are representative of three similar experiments. The data are the means ± standard deviations for three determinations. *, $P < 0.05$ for the Δ*ply* mutant versus rPLY plus the Δ*ply* mutant.

Δ*ply* mutant up to an MOI of 50 did not result in such an enhancement (data not shown). These findings clearly indicate that the PLY-dependent cytokine response is enhanced by other stimuli from bacterial cells but that PLY, not any other bacterial components, is solely responsible for the induction of IL-1α, IL-1β, and IL-18 production, which requires the activation of cleaving enzymes, including caspase-1. However, costimulation with rPLY and purified TLR agonists, such as LPS or $Pam_3CSK_4$, resulted in no marked enhancement of the

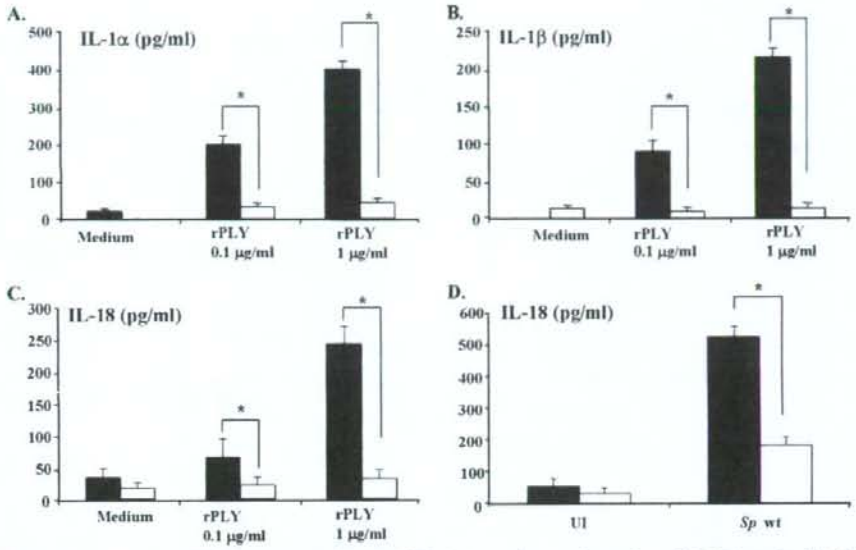


FIG. 5. TLR4-dependent induction of inflammatory cytokines by PLY. Peritoneal macrophages from C57BL/6 wt mice (black bars) and TLR4 KO mice (white bars) were stimulated with cholesterol-treated rPLY (A to C) or infected with wt *S. pneumoniae* at a macrophage/bacterium ratio of 1:10 (D) for 24 h, and the culture supernatants were then collected. The amounts of IL-1α, IL-1β, and IL-18 in culture supernatants were determined by ELISA. The results are representative of three similar experiments. The data are the means ± standard deviations for three determinations. Asterisks indicate that the value is significantly different from that of wt cells ($P < 0.05$).

production of IL-1α, IL-1β, and IL-18 (data not shown), suggesting that the mechanism of enhancement might be more complex than just a simultaneous stimulation with rPLY and TLR agonists from *S. pneumoniae*.

**Critical role of TLR4 in IL-1α, IL-1β, and IL-18 production induced by PLY.** In a previous study, it was shown that PLY induces TNF-α and IL-6 production through TLR4 (27). To investigate whether TLR4 is also required for the production of IL-1α, IL-1β, and IL-18 induced by rPLY, we compared the levels of these cytokines produced by macrophages from C57BL/6 background TLR4-deficient mice and wt mice in response to rPLY. In addition to that of TNF-α and IL-6, the production of IL-1α, IL-1β, and IL-18 induced by rPLY was dependent on TLR4, as these cytokines were produced only by wt macrophages upon stimulation with rPLY (Fig. 5A to C). The production of IL-18 induced by infection with wt *S. pneumoniae* was also critically dependent on the presence of TLR4, suggesting that production of the cytokine in response to *S. pneumoniae* is due to the recognition of PLY via TLR4 (Fig. 5D). Because the production of IL-1α, IL-1β, and IL-18 is regulated both transcriptionally and posttranscriptionally, we next examined the involvement of TLR4 in the rPLY-induced gene expression of these cytokines. rPLY induced or up-regulated the expression of IL-1α, IL-1β, and IL-18 in macrophages from wt mice but not in those from TLR4 KO mice (Fig. 6). These results indicate that TLR4 plays a role in the production of IL-1α, IL-1β, and IL-18 induced by rPLY, at least at the gene expression level. In addition, the difference between rPLY and LPS in inducing the production of IL-1α, IL-1β, and IL-18 resulted from a posttranscriptional process, not the ability to induce gene expression, because LPS induced gene expression of these cytokines at levels comparable to those induced by rPLY.

**Activation of TLR4 downstream signals by rPLY.** As shown in Fig. 2 and 6, there was dissociation between the gene expression and production of TNF-α after stimulation of macrophages with rPLY. Moreover, the levels of rPLY-induced gene

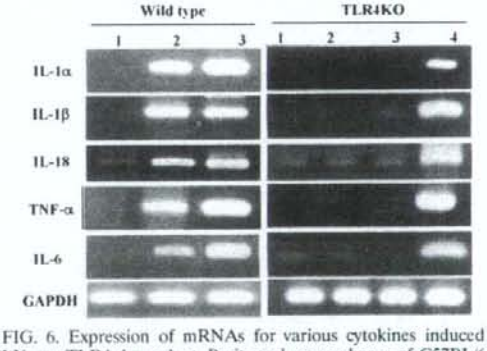


FIG. 6. Expression of mRNAs for various cytokines induced by rPLY was TLR4 dependent. Peritoneal macrophages of C57BL/6 wt and TLR4 KO mice were stimulated with rPLY, LPS, and $Pam_3CSK_4$ (1 μg/ml) for 6 h. Total RNA was extracted and subjected to reverse transcription-PCR for detection of cytokine mRNAs for IL-1α, IL-1β, IL-18, TNF-α, IL-6, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Lanes: 1, PBS; 2, rPLY; 3, LPS; 4, $Pam_3CSK_4$. Representative results are shown. Similar results were obtained in three separate experiments.

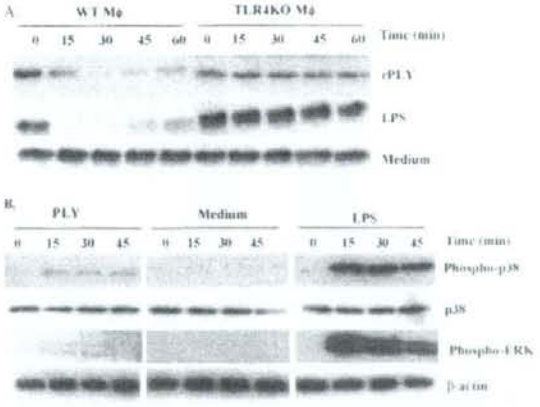


FIG. 7. Activation of TLR4 downstream signals by rPLY. Peritoneal macrophages from C57BL/6 (WT) and TLR4 KO mice were stimulated with medium alone, rPLY, or LPS (1 μg/ml). (A) At the indicated times, cell lysates were collected and IκBα degradation was analyzed by Western blotting. (B) The cell lysates described in panel A were then subjected to Western blotting using antibodies specific to p38, phospho-p38, phospho-ERK, and β-actin. The results are representative of at least three independent experiments.

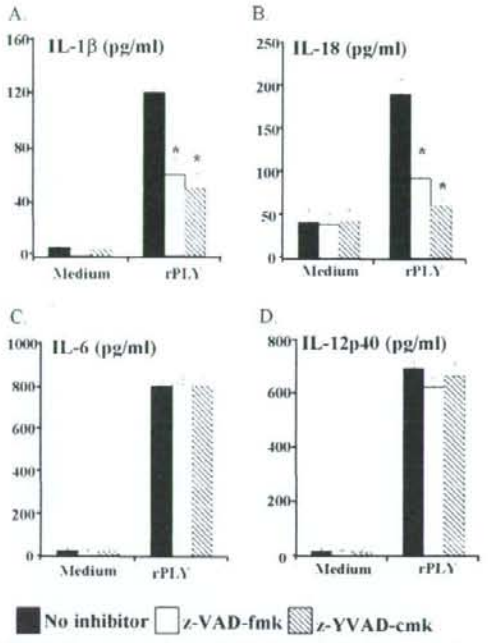


FIG. 8. Pneumolysin-induced IL-1β and IL-18 production is caspase-1 dependent. Peritoneal macrophages were stimulated with cholesterol-treated rPLY (1 μg/ml) in the presence or absence of a broad-spectrum caspase inhibitor (z-VAD-fmk [30 μM]) and a caspase-1-specific inhibitor (z-YVAD-cmk [30 μM]) for 24 h, and the culture supernatant was collected. The amounts of IL-1β (A), IL-18 (B), IL-6 (C), and IL-12p40 (D) were determined by ELISA. The results are representative of three separate experiments. The data are the means ± standard deviations for three or four determinations. *, $P < 0.05$ compared to no-inhibitor treatment.

expression and production of TNF-α and IL-6 were significantly lower than those induced by LPS (Fig. 2 and 6). These findings may be due to some difference in the events downstream of TLR4 ligation with rPLY or LPS. Therefore, we first examined the proteolysis of IκBα, a regulator of NF-κB, to estimate the activation of NF-κB. Both rPLY and LPS induced the degradation of IκBα, but some differences were observed in its strength and kinetics. The LPS-induced degradation of IκBα was observed as early as 5 min after stimulation (data not shown), whereas that induced by rPLY took place later (Fig. 7A). The degradation of IκBα was not observed in TLR4-deficient macrophages after stimulation with both rPLY and LPS. Next, the activation of MAPKs was compared. LPS strongly induced the phosphorylation of p38 MAPK and extracellular signal-regulated kinases (ERKs). In contrast, rPLY induced only weak phosphorylation of p38 MAPK and hardly induced ERK phosphorylation (Fig. 7B). These results indicate that the activation of the signaling process after sensing by TLR4 differs between stimulation with rPLY and LPS, which may account for the different profiles of the cytokine response to stimulation with these two TLR4 agonists.

**Caspase-1 dependency of PLY-induced IL-1β and IL-18 production.** Intracellular IL-1β and IL-18 are not secreted from cells as active forms until they are cleaved by caspase-1. To understand whether PLY-induced macrophage production of IL-1β and IL-18 is due to the activation of caspase-1, the effects of caspase inhibitors were examined. Peritoneal macrophages were pretreated with the caspase-1-specific inhibitor z-YVAD-cmk or the broad-spectrum caspase inhibitor z-VAD-fmk. Both inhibitors effectively reduced the secretion of IL-1β and IL-18 in the culture supernatant, suggesting that caspase-1 activation was induced by rPLY stimulation (Fig. 8). Nonspecific effects of the two inhibitors could be ruled out because the production of IL-6 and IL-12p40 was unaffected by either inhibitor.

**Activation of caspase-1 by PLY.** Since the production of IL-1β and IL-18 induced by rPLY was dependent on caspase-1, to confirm the PLY-induced caspase activation, we first tried to detect the active form of caspase-1 in macrophages stimulated with rPLY by means of Western blotting using a polyclonal antibody against the cleaved p20 fragment of caspase-1. This approach was not successful, however, because the amount of p20 fragment inside cells was too small, probably due to a rapid secretion of the active form soon after the cleavage (28). For this reason, we concentrated intracellular activated caspase-1 by a pull-down method using biotinylated YVAD-cmk, which binds to the active form of caspase-1, as previously described, with slight modification (29), and then applied the samples to Western blotting. The activated form of caspase-1 could be detected in macrophages stimulated by both cholesterol-treated rPLY and untreated rPLY (Fig. 9A). Caspase-1 activation was induced sufficiently with even a low dose (0.1 μg/ml) of rPLY, whereas 1 μg/ml LPS was not capable of inducing caspase-1 activation. As previously reported (47), an additional stimulation with ATP was required for the activation of caspase-1 in macrophages stimulated with LPS (Fig. 9B). To