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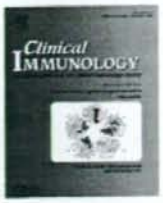

Disruption of Nrf2 enhances susceptibility to airway inflammatory responses induced by low-dose diesel exhaust particles in mice

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Mouse model

Abstract To test our hypothesis that diesel exhaust particle (DEP)-induced oxidative stress and host antioxidant responses play a key role in the development of DEP-induced airway inflammatory diseases, C57BL/6 nuclear erythroid 2 P45-related factor 2 (Nrf2) knockout ($Nrf2^{-/-}$) and wild-type mice were exposed to low-dose DEP for 7 h/day, 5 days/week, for 8 weeks. $Nrf2^{-/-}$ mice exposed to low-dose DEP showed significantly increased airway hyperresponsiveness and counts of lymphocytes and eosinophils, together with increased concentrations of IL-12 and IL-13, and thymus and activation-regulated chemokine (TARC), in BAL fluid than wild-type mice. In contrast, expression of antioxidant enzyme genes was significantly higher in wild-type mice than in $Nrf2^{-/-}$ mice. We have first demonstrated that disruption of Nrf2 enhances 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 some role in the development of DEP-induced airway inflammation.

Introduction

Air pollution is associated with increased mortality and morbidity. Although air pollutants include both gaseous components (ozone, carbon monoxide, sulfur dioxide, and nitrogen dioxide) and particulate components, it has been shown that particulates, particularly PM10 (particulate matter with diameter less than 10 μm), are more relevant to many disorders. Currently, much attention is being focused on PM2.5 in relation to adverse health effects. Diesel exhaust particles (DEP) are the major component of PM2.5, and therefore the relationship between PM2.5 or PM10 and some diseases has been investigated

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intensively [1–5]. The roles of reactive oxygen species (ROS) generated by exposure to DEP and the subsequent generation of the oxidative stress response have been emphasized through *in vitro* experimental studies [6–11]. These findings suggest that DEP induce activation of transcription factors and that ROS may play an important role in these processes. Thus, imbalance between oxygen stress/proinflammatory proteins and antioxidant proteins may be a key player in the development of hazardous effects. However, it is still not fully clear whether oxidative stress caused by inhalation of DEP leads to airway inflammation and airway hyperresponsiveness *in vivo*.

We have shown previously that continuous low-level exposure to DEP (100 $\mu g/m^3$ for 7 h/day, 5 days/week) significantly augments AHR and Th2-type cytokine/chemokine gene expression in murine asthma models [12]. Studies using two different mouse strains have demonstrated a difference in susceptibility to DEP exposure between them, and suggested that certain antioxidant enzymes may play an important role in susceptibility, C57BL/6 mice being more sensitive to low-dose DEP exposure than BALB/c mice [13,14].

Nuclear erythroid 2 P45-related factor 2 (Nrf2) is a redox-sensitive basic leucine zipper transcription factor that is involved in the transcriptional regulation of many antioxidant genes. The Nrf2-regulated genes in the lungs include almost all of the relevant antioxidant enzymes, such as HO-1 and several members of the GST family [15]. Therefore, to clarify whether or not oxidative stress and host antioxidant defenses play a central role in the pathogenesis of lung disease, several disease models using $Nrf2^{-/-}$ mice have been studied, including ovalbumin (OVA)-induced asthma [16], bleomycin-induced lung fibrosis [17], and cigarette smoke-induced emphysema [18]. It is reported that oxidative stress is involved in the development of DEP-induced airway inflammation [6–11] and that Nrf2 is also a key transcription factor that regulates antioxidant and defense action against the proinflammatory and oxidizing effects of DEP *in vitro* [9,11]. Furthermore, DNA adduct formation has been shown to be accelerated in the lungs of $Nrf2^{-/-}$ mice exposed to DEP (3 mg/m^3 for 4 weeks) [19]. However, no study has yet utilized $Nrf2^{-/-}$ mice to examine the pathogenesis of airway inflammation induced by low-dose DEP.

To test the hypothesis that DEP-induced oxidative stress and host antioxidant responses play a key role in the development of DEP-induced airway inflammatory disease *in vivo*, we conducted the present study using C57BL/6 $Nrf2^{-/-}$ and $Nrf2^{+/+}$ mice, and examined airway inflammatory responses and host antioxidant responses to low-dose DEP (i.e., at concentrations similar to those inhaled outdoors) exposure.

Materials and methods

Animals

Nrf2-deficient C57BL/6 mice were generated as described by Itoh et al. [20]. Mice were genotyped for Nrf2 status by PCR amplification of genomic DNA extracted from the tail [21]. PCR amplification was performed using three different primers:

Nrf2-sense for both genotypes: 5′-TGGACGGGACTATTGAAGGCTG-3′

Nrf2-antisense for wild-type mice: 5′-GCCGCCTTTTCAGTAGATGGAGG-3′

Nrf2-antisense for LacZ: 5′-GCGGATTGACCGTAATGGGATAGG-3′.

Amplification was performed using 30 cycles of 96 °C 20 s, 59 °C 30 s, and 72 °C 45 s. The wild-type allele produces a 734-bp band, while the knockout allele produces a 449-bp band. Mice were housed under specific pathogen-free (SPF) conditions with controlled temperature and lighting (23 ± 2 °C, 12 h light/dark periods). Age-matched 6-week-old female mice from the same litter were placed into chambers under same controlled conditions and exposed to DEP. All procedures conformed to the National Institutes of Health (NIH) guidelines for the care and use of laboratory animals.

DEP exposure

The *in vivo* DEP exposure system has been described previously [22,23]. The concentration of DEP was monitored and kept low (approximately 100 $\mu g/m^3$). C57BL/6 $Nrf2^{-/-}$ and $Nrf2^{+/+}$ mice were exposed to DEP for 7 h daily, 5 days a week.

Experimental protocol

$Nrf2^{-/-}$ and $Nrf2^{+/+}$ C57BL/6 mice were exposed to low-dose DEP or clean air (SPF) for 8 weeks. AHR was measured in all experimental groups immediately, and mice were sacrificed on day 56 of exposure to DEP or clean air in all experimental groups. We examined the histopathology of the lung tissues and the cell populations in BAL fluid. We also measured the concentrations of inflammatory cytokines and chemokines in the BAL fluid and the IgE, IgG_1 and IgG_{2a} levels in the serum. We also examined the gene expression of antioxidants in the lung tissues.

Determination of AHR

Airway hyperresponsiveness was assessed by whole-body plethysmography with a free-moving application (Buxco Electronics, Troy, NY) [24] in accordance with a previously published procedure [12,13].

Histological analysis

For histologic examination, 10% formalin-fixed lung tissues were embedded in paraffin, cut into sections, and stained with hematoxylin and eosin (HE), and periodic acid-Schiff (PAS). Histopathological changes were examined using a light microscope (Eclipse E800, Nikon, Tokyo, Japan).

BAL and cell differentials in BAL fluid

BAL was performed as described previously [13,14], and the total number of cells in the BAL fluid was counted with a hemocytometer. For differential counts of leukocytes in BAL fluid, cytospin smear slides (Lab Systems; Tokyo, Japan) were prepared and stained with Diff-Quick Romanowski stain (Muto Kagaku Co., Tokyo, Japan).

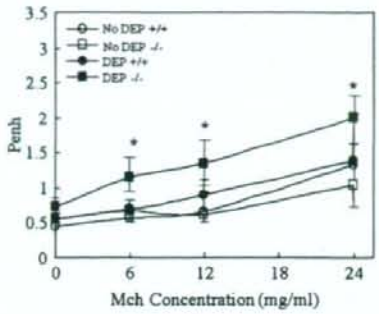

Figure 1 Airway hyperresponsiveness was assessed by whole-body plethysmography with free-moving application and then evaluated by Penh values in response to inhaled aerosolized methacholine (0, 6, 12, 24 mg/ml). The *X* axis shows the concentration of methacholine (mg/ml); the *Y* axis shows the values of Penh. $^{+/+}$, wild-type mice; $^{-/-}$, Nrf2 knockout mice. Results are means ± SD of data in each group (n = 16). *p<0.05 $Nrf2^{-/-}$ mice vs $Nrf2^{+/+}$ mice at each methacholine concentration point.

Measurement of cytokines/chemokines in BAL fluid

Immunoreactivity for IL-12, IL-4, IL-13, MCP-1, eotaxin, RANTES, and TARC in the BAL fluid supernatants was measured with an enzyme-linked immunosorbent assay (ELISA) kit (Biosource International, Inc., Camarillo, CA). ELISA was carried out in accordance with the manufacturer's instruction sheet. Each sample was assayed in triplicate.

Measurement of IgG_1, IgG_{2a}, and IgE in serum

Immunoreactivity for IgG_1, IgG_{2a} (Cygnus Technologies, Inc., Southport, NC), and IgE (Bethyl Laboratories, Inc., Montgomery, TX) in the serum was measured with an ELISA kit in accordance with the manufacturer's instruction sheet. Each sample was assayed in triplicate.

Quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from the lung tissues with TRIzol Reagent (Gibco BRL, Gaithersburg, MD) in accordance with the manufacturer's instructions. Complementary DNA (cDNA) was synthesized as described previously [25] and quantified with a sequence detector (7900HT Sequence Detection System; Applied Biosystems) using PCR Master Mix and the respective inventoried primers including a β-actin control (TaqMan Universal PCR Master Mix, Applied Biosystems). TaqMan assays were repeated in triplicate samples for each of the selected antioxidant enzyme genes in each lung sample. The mRNA expression levels for all samples were normalized to the level of the housekeeping gene β-actin. Selected antioxidant enzyme genes and their assay ID were as follows: glutamate-cysteine ligase, modifier subunit (GCLm, Mm00514996_m1), glutamate-cysteine ligase, catalytic subunit (GCLc, Mm00802655_m1), glucose-6-phosphate dehydrogenase X-linked (G6PD, Mm00656735_g1), glutathione-S-transferase, alpha3 (GST-α3, Mm00494798_m1), glutathione-S-transferase m1 (GST-M1, Mm00833915_g1), glutathione-S-transferase pi2 (GST-P2, Mm00839138_g1), heme-oxygenase-1 (HO-1, Mm00516004_m1), superoxide dismutase 2 (SOD2, Mm00449726_m1), glutathione reductase 1 (GSr, Mm00833903_m1), and β-actin (Mm00607939_s1).

Statistical analysis

Results are shown as means ± standard deviation (SD). Differences between groups were determined by Student's *t* test using the Stat Mate III software package (ATMS Digitals Medical Station, Tokyo, Japan). Differences at $p<0.05$ were considered significant.

Results

Assessment of changes in AHR in response to DEP exposure

To examine airway responses to low-dose DEP in $Nrf2^{-/-}$ mice, we first assessed AHR (as expressed in Penh) using whole-body plethysmography.

Exposure to DEP significantly increased the airway reactivity to methacholine (6, 12, and 24 mg/ml) in $Nrf2^{-/-}$ mice compared with $Nrf2^{+/+}$ mice (Fig. 1).

Lung histopathology

The histological specimens revealed no change in response to DEP exposure in $Nrf2^{+/+}$ mice (Fig. 2), but PAS staining-positive mucus cell hyperplasia was evident in $Nrf2^{-/-}$ mice (Fig. 2B). There were no inflammatory cell infiltrates in either $Nrf2^{+/+}$ or $Nrf2^{-/-}$ mice.

BAL cell differentials

Examination of the BAL fluid showed that the total number of cells and the number of macrophages were significantly lower in $Nrf2^{-/-}$ mice than in $Nrf2^{+/+}$ mice. The numbers of lymphocytes and eosinophils in the BAL fluid after DEP exposure were significantly higher in $Nrf2^{-/-}$ mice than in $Nrf2^{+/+}$ mice (Fig. 3).

Cytokine/chemokine levels in BAL fluid

The levels of IL-12 and IL-13 in BAL fluid after DEP exposure were significantly higher in $Nrf2^{-/-}$ mice. The level of IL-4 in BAL fluid did not change significantly after DEP exposure in either $Nrf2^{+/+}$ or $Nrf2^{-/-}$ mice (Fig. 4A). The level of TARC in BAL fluid after DEP exposure was significantly greater in $Nrf2^{-/-}$ mice than in $Nrf2^{+/+}$ mice. The levels of monocyte chemoattractant protein (MCP)-1, regulated upon activation, normal T expressed and secreted (RANTES) and Eotaxin in tBAL fluid were not changed significantly after DEP exposure in either $Nrf2^{+/+}$ or $Nrf2^{-/-}$ mice (Fig. 4B).

IgG_1, IgG_{2a}, and IgE levels in serum

There were no significant changes in the levels of IgE, IgG_1 and IgG_{2a} in serum after DEP exposure in either $Nrf2^{+/+}$ or $Nrf2^{-/-}$ mice (Fig. 5).

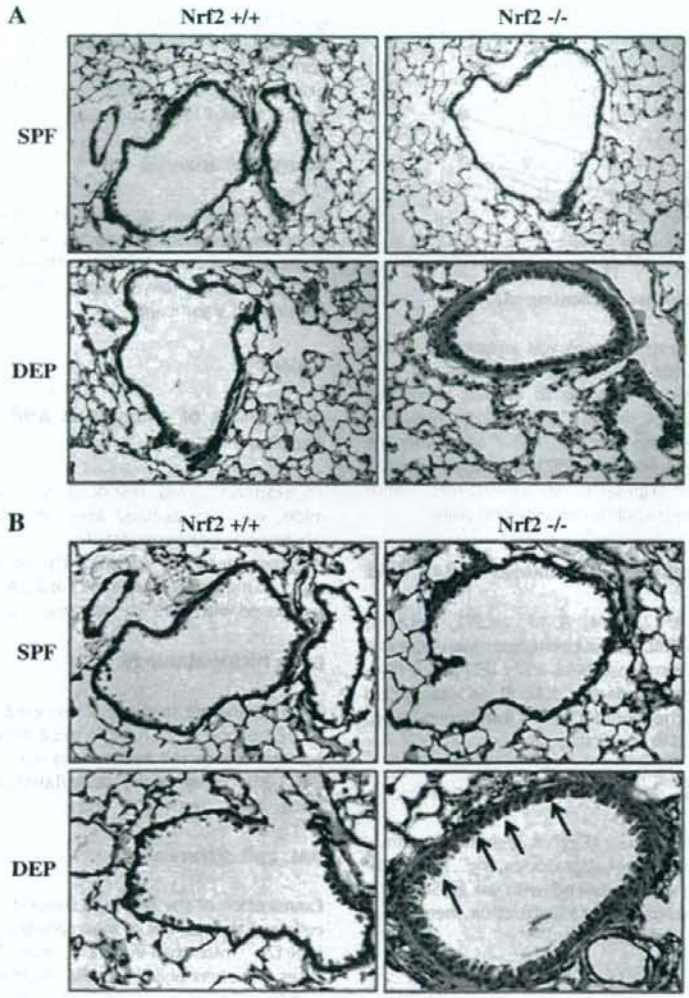

Figure 2 Histopathology of the lung tissues. Lung sections stained with HE and examined by light microscopy (100×) (A). Lung sections stained with PAS and examined by light microscopy (200×) (B). The arrows indicate the PAS-positive cells.

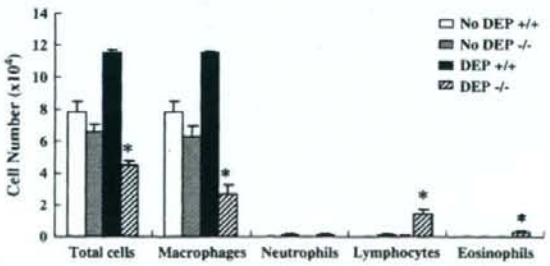

Figure 3 Changes in total cells and differentials in BAL fluid. The smear preparations in BAL fluid were stained with Diff-Quick Romanowski stain. Results are means ± SD of data in each group ($n=6$). *$p<0.05$ $Nrf2^{-/-}$ mice vs $Nrf2^{+/+}$ mice.

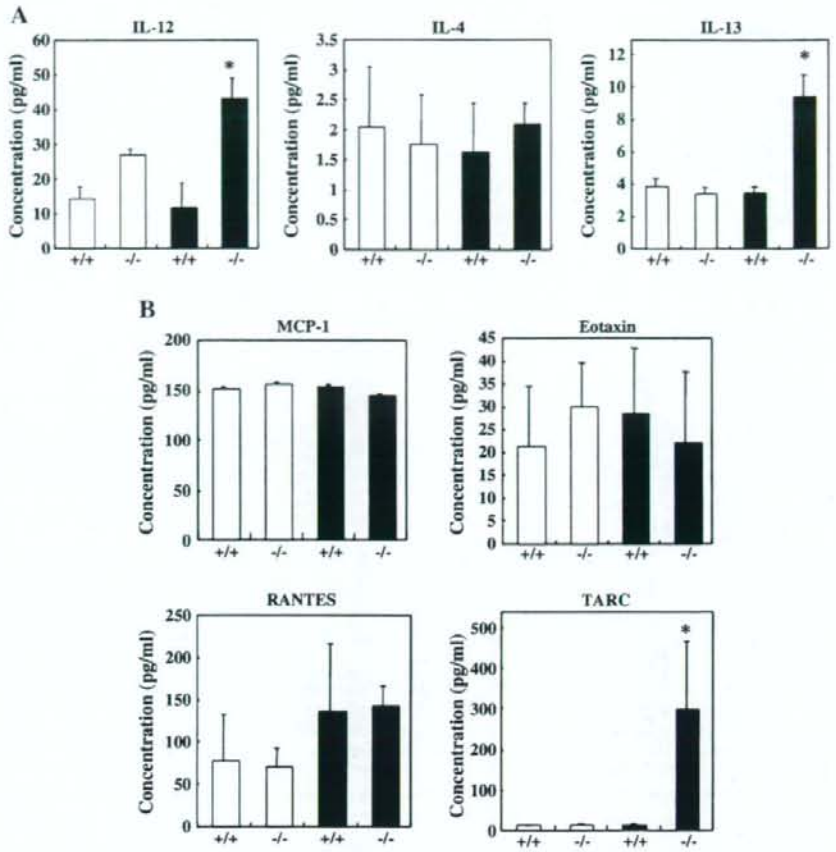

Figure 4 Cytokine (A) and chemokine (B) levels in BAL fluid as evaluated by ELISA. Clear bar, no DEP (not exposed to DEP) group; solid bar, DEP group; $^{+/+}$, wild-type mice; $^{-/-}$, Nrf2 knockout mice. Results are means ± SD of data in each group ($n=6$). *$p<0.05$ $Nrf2^{-/-}$ mice vs $Nrf2^{+/+}$ mice.

Induction of antioxidant enzyme genes in lung tissues

Changes in the expression of mRNA for various antioxidant enzymes were determined by real RT-PCR. After DEP exposure, the respective fold changes in mRNA expression in the lungs of $Nrf2^{+/+}$ and $Nrf2^{-/-}$ mice were: GCLm (3.4 vs 1.8), GCLc (5.5 vs 2.1), G6PD (18 vs 7.8), GST-α3 (7.4 vs 1.6), GST-M1 (6.2 vs 1.3), GST-P2 (6.4 vs 2.1), HO-1 (11.8 vs 6.9), SOD2 (17 vs 9.1), and GSr (8.8 vs 5.9) (Fig. 6). Thus, gene

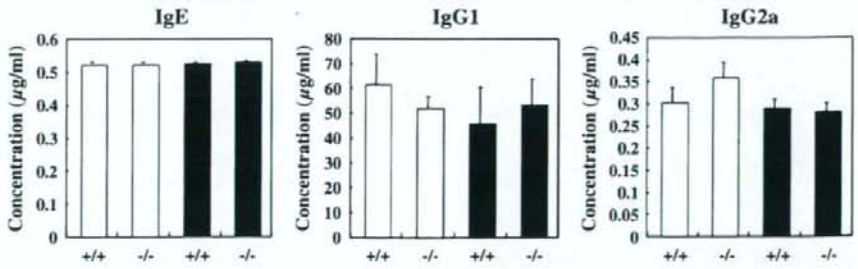

Figure 5 Immunoglobulin levels in sera evaluated by ELISA. Clear bar, no DEP (not exposed to DEP) group; solid bar, DEP group; $^{+/+}$, wild-type mice; $^{-/-}$, Nrf2 knockout mice. Results are means ± SD of data in each group ($n=6$).

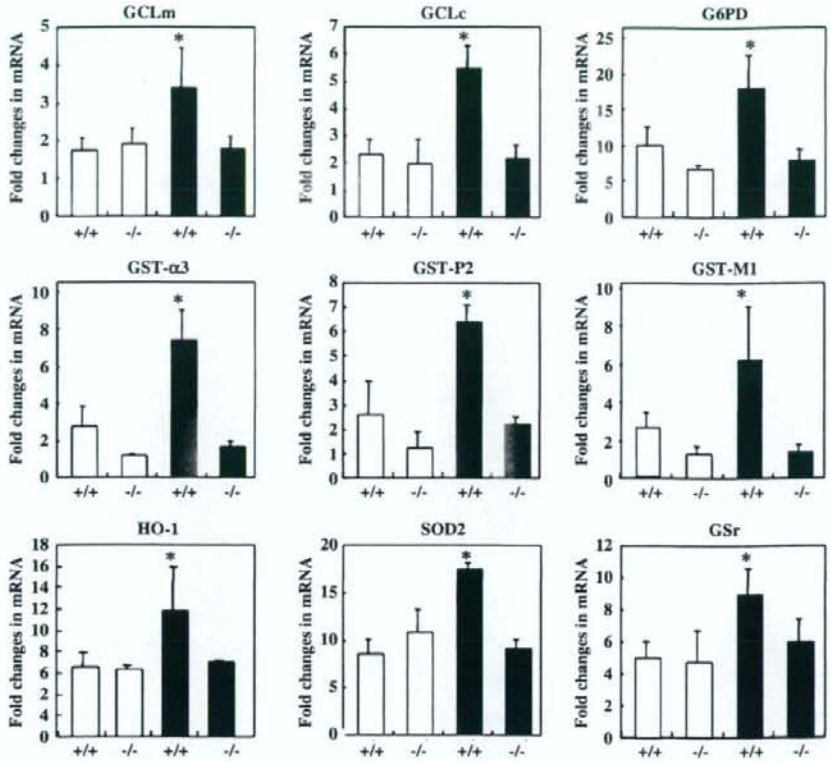

Figure 6 Real-time RT-PCR for genes of various antioxidants in the lungs. Clear bar, no DEP (not exposed to DEP) group; solid bar, DEP group; $^{+/+}$, wild-type mice; $^{-/-}$, Nrf2 knockout mice. Results are means ± SD of data in each group ($n = 3$). *$p < 0.05$ $Nrf2^{-/-}$ mice vs $Nrf2^{+/+}$ mice.

expression of various antioxidant enzymes was significantly increased in $Nrf2^{+/+}$ mice compared with $Nrf2^{-/-}$ mice.

Discussion

The present study using Nrf2 gene knockout mice revealed for the first time that disruption of the Nrf2 gene enhanced susceptibility to airway inflammatory responses induced by inhalation of low-dose DEP. $Nrf2^{-/-}$ and $Nrf2^{+/+}$ C57BL/6 mice were exposed to low-dose DEP (i.e., at concentrations similar to those inhaled outdoors) for 8 weeks, and in $Nrf2^{-/-}$ mice significant increases of AHR, numbers of lymphocytes and eosinophils, and levels of IL-12, IL-13 and TARC in BAL fluid were observed in comparison with those of $Nrf2^{+/+}$ mice. In contrast, gene expression of antioxidant enzymes was significantly increased in $Nrf2^{+/+}$ mice in comparison with $Nrf2^{-/-}$ mice. As the magnitude of gene induction of these antioxidant enzymes was considerably higher in $Nrf2^{+/+}$ than in $Nrf2^{-/-}$ mice, it was clearly associated with the activation of Nrf2 in response to DEP-induced lung inflammation.

We assessed airway hyperresponsiveness (as expressed by enhanced Pause values (Penh)) by whole-body plethysmography. Penh has been used in an experimental mouse model to evaluate airway hyperresponsiveness [26–28]. Exposure to DEP increased airway reactivity to methacholine more significantly in $Nrf2^{-/-}$ mice than in $Nrf2^{+/+}$ mice. Our previous study showed that the changes in Penh induced by methacholine were significantly greater 1 week after DEP exposure in C57BL/6 mice, but that Penh recovered to the baseline level by 8 weeks after DEP exposure, and the changes in AHR were independent of the airway inflammation caused by oxidative stress derived from low-dose DEP exposure [13]. The current data suggest that AHR may increase with oxidative stress induced by continuous, long-term (longer than 8 weeks) exposure to DEP in wild-type mice. PAS-positive mucus cell hyperplasia was found only in $Nrf2^{-/-}$ mice, without destruction of the airway epithelial cells in the lung tissues. Although it has been confirmed that AHR increases with oxidative stress induced by DEP exposure, the mechanism by which AHR increases in $Nrf2^{-/-}$ mice is still unclear. It is speculated that, in this system, the changes in AHR caused by low-dose DEP may depend on the genetic strain of mouse employed, as reported by Depuydt et al. [29]. Our results suggest that Nrf2 plays a key role in regulation of AHR in oxidative stress caused by DEP exposure.

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.

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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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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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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 μl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 μl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 μl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 μl（Signal probe/Invader oligonucleotide/FRET probe溶液2.6 μlとCleavase酵素0.4 μl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 μl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 μl，8mM dNTP 1 μl，プライマー2 μl，滅菌蒸留水40.5 μl，AmpliTaq Gold（アプライドバイオシステムズ）0.5 μl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E4株，パターンF1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。

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