Complementary DNA Microarray Analysis in Acute Lung Injury Induced by Lipopolysaccharide and Diesel Exhaust Particles

RIE YANAGISAWA,* HIROHISA TAKANO,*,¶,[1] KEN-ICHIRO INOUE,* TAKAMICHI ICHINOSE,† SEI-ICHI YOSHIDA,† KAORI SADAKANE,† KEN TAKEDA,‡ SHIN YOSHINO,§ KOUYA YAMAKI,§ YOSHITO KUMAGAI,|| AND TOSHIKAZU YOSHIKAWA¶

*Pathophysiology Research Team, National Institute for Environmental Studies, Ibaraki, Japan; †Department of Health Sciences, Oita University of Nursing and Health Sciences, Oita, Japan; ‡Department of Hygiene Chemistry, Faculty of Pharmaceutical Sciences, Tokyo University of Science, Chiba, Japan; §Department of Pharmacology, Kobe Pharmaceutical University, Kobe, Japan; ||Department of Environmental Medicine, Institute of Community Medicine, University of Tsukuba, Ibaraki, Japan; and ¶Inflammation and Immunology, Kyoto Prefectural University of Medicine, Kyoto, Japan.

We have recently shown that diesel exhaust particles (DEP) synergistically enhance acute lung injury related to lipopolysaccharide (LPS) in mice. The present study used cDNA microarray to elucidate the effects of DEP on the global pattern of LPS-related gene expression in the murine lung. The number of genes upregulated ≥2-fold as compared with their expression levels in the vehicle group was greater in the LPS group than in other groups, but treatment with DEP and LPS dramatically increased the number of the genes upregulated ≥6-fold. In particular, gene expression of metallothionein-1 and -2, S100 calcium-binding protein A9, lipocalin 2, and small inducible cytokine B family member 10 was higher by ≥20-fold in the DEP + LPS group than in the vehicle group. These results were concomitant with those obtained by real-time reverse transcription–polymerase chain reaction analysis in the overall trend. Our findings suggest that intense, focused expression of genes such as S100 calcium-binding protein A9, lipocalin 2, and small inducible cytokine B family member 10 relates to the synergistic aggravation of acute lung injury by LPS and DEP rather than weak, broad expression of various genes by exposure of LPS alone. Exp Biol Med 229:1081–1087, 2004.

Key words: acute lung injury; cDNA microarray; lipopolysaccharide; diesel exhaust particles

[1] To whom correspondence should be addressed at Pathophysiology Research Team, National Institute for Environmental Studies, 16-2 Onogawa, Tsukuba, Ibaraki, 305-8506, Japan. E-mail: htakano@nies.go.jp.

Received March 5, 2004.
Accepted August 2, 2004.

1535-3702/04/22910-1081$15.00

Introduction

Previous epidemiologic studies have demonstrated that exposure to ambient particulate matter (PM) is positively associated with increases in the morbidity and daily mortality caused by respiratory diseases (1, 2). Diesel exhaust particles (DEP) are main constituents of PM 2.5, which are PM with <2.5-μm diameter. DEP affect a variety of respiratory diseases, including asthma (3), pulmonary edema (4), and lung cancer (5). Our previous studies have shown that intratracheal inoculation of DEP enhances antigen-specific IgG1 production, eosinophilic airway inflammation, and the expression of cytokines in the murine lung (6, 7).

Lipopolysaccharide (LPS), a component of the cell wall of gram-negative bacteria, induces or exacerbates a variety of lung diseases, including asthma (8) and acute lung injury (9). Exposure of rodents to LPS recruits neutrophils and increases the expression of proinflammatory molecules (10, 11). Exposure to DEP with LPS or preincubation with LPS before DEP treatment increases interleukin (IL)-1β secretion in peripheral blood mononuclear cells (12). We have recently shown that DEP synergistically enhance neutrophilic lung inflammation related to LPS in mice (13). This synergistic enhancement has been concomitant with the increased gene expression of proinflammatory molecules such as IL-1β and macrophage inflammatory protein 1α (MIP-1α). However, this study has generally been limited to the analysis of only a few genes and therefore has provided little information about the global pattern of gene expression related to DEP in the presence or absence of LPS.

cDNA microarray or DNA-chip technology provides rapid analysis and abundance of information on expressed gene. Previous studies using cDNA microarrays have found that exposure to DEP contributes to pulmonary carcinogenesis in rat lungs (14), to the repair of DNA damage, and to cell proliferation in rat alveolar macrophages (15). However, no previous study has examined the global pattern of gene expression that follows DEP enhancement of inflammatory lung injury.

In the present study, we used cDNA microarray technology to elucidate the effects of DEP on the global pattern of gene expression related to LPS in the murine lung. The results were further confirmed by using real-time transcription–polymerase chain reaction (RT-PCR) analysis. Our studies showed that DEP in the presence of LPS markedly enhanced the expression of five genes, which might be responsible for the synergistic aggravation of LPS-induced acute lung injury.

Materials and Methods

Animals. We used Jcl:ICR male mice (6 weeks old) weighing 29–33 g (Japan Clea Co., Tokyo, Japan). They were fed and housed as previously described (7). The studies followed the National Institutes of Health guidelines for the experimental use of animals. The Institutional Review Board approved all animal studies.

Collection of DEP. A 4JB1-type, light-duty, four-cylinder, 2.74-L, Isuzu diesel engine (Isuzu Automobile Co., Tokyo, Japan) under computer control was connected to a dynamometer (Meiden-sha, Tokyo, Japan). The engine was operated on standard diesel fuel at 1500 rpm under a load of 10 torques (kg/m). DEP were collected as previously described (16). The mass median aerodynamic diameter of DEP was 0.4 μm by analysis of an Anderson Air Sampler (Shibata Science Technology, Tokyo, Japan). Most of the particles were globular in shape.

Study Protocol. The mice were randomly allocated to four experimental groups. The vehicle group received 1× phosphate-buffered saline (PBS) at pH 7.4 (Invitrogen Corp., Carlsbad, CA) containing 0.025% Tween 80 (Nacalai Tesque, Kyoto, Japan). The DEP group received 250 μg DEP in the same vehicle. The LPS (*Escherichia coli* B55: 05; Difco Lab, Detroit, MI) group received 100 μg LPS in the same vehicle, the DEP + LPS group received both 250 μg DEP and 100 μg LPS in the same vehicle, and the suspensions were sonicated for 2 mins with an ultrasonic disruptor (UD-201; Tomy Seiko, Tokyo, Japan). Each group of mice was anesthetized with 4% halothane (Takeda Chemical Industries Ltd., Osaka, Japan) and then intratracheally inoculated with 100-μl aliquots via a polyethylene tube. The endotoxin activity, which was determined by Limulus Amebocyte Lysate assay (Seikagaku-kogyo, Tokyo, Japan), was lower than the detection limit (0.001 EU/ml) in the vehicle solutions and DEP solutions ($n = 8$ in each solution).

Extraction of mRNA. Total RNAs were extracted with ISOGEN (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. Poly(A)$^+$mRNA for cDNA microarray analysis was prepared by using Oligotex-dT30 mRNA Purification Kits (TaKaRa, Kyoto, Japan) according to the manufacturer's protocol.

Fluorescent Labeling of Probe. Isolated mRNA was reverse transcribed with 5′Cy3 or Cy5 labeled random 9-mers (Operon Technologies Inc., Alameda, CA). Reaction mixtures were incubated for 2 hrs at 37°C with 200 ng polyA RNA, 200 units Moloney murine leukemia virus reverse transcriptase (Invitrogen), 4 m*M* dithiothreitol (DTT), 1 unit RNase Inhibitor (Ambion, Austin, TX), 0.5 m*M* deoxynucleotide triphosphates (dNTPs), and 2 μg labeled 9-mers in 25-μl volume with enzyme buffer supplied by the manufacturer (Invitrogen). The reactions were terminated by incubation at 85°C for 5 mins. The paired reactions were combined and purified with a TE-30 column (Clontech, Palo Alto, CA), brought to 90 μl with dH_2O, and then precipitated with 2 μl of 1 mg/ml glycogen, 60 μl 5*M* NH_4OAc, and 300 μl ethanol. After centrifugation the supernatant was decanted and the pellet was resuspended in 24 μl of hybridization buffer, which consisted of 5X SSC, 0.2% sodium dodecyl sulfate, 1 m*M* DTT.

Hybridization and Scanning. Hybridization was carried out by using the LifeArray (Incyte Pharmaceuticals Inc., Palo Alto, CA). The LifeArray contained 9596 mouse cDNA spots, which consisted of 6238 named genes and 3358 expressed sequence tags. Probe solutions were thoroughly resuspended by incubating at 65°C for 5 mins with mixing. The probe was applied to the array and covered with a 22-mm^2 glass coverslip and placed in a sealed chamber to prevent evaporation. After hybridization at 60°C for 6.5 hrs, slides were washed in three consecutive washes of decreasing ionic strength. Microarrays were scanned in both Cy3 and Cy5 channels with Axon GenePix scanners (Foster City, CA) with a 10-mm resolution. The signal was converted into 16-bits-per-pixel resolution, yielding a 65,536-count dynamic range.

Normalization and Ratio Determination. Incyte GEMtools software (Incyte Pharmaceuticals) was used for image analysis. The element was determined by a gridding and region detection algorithm. The area surrounding each element image was used to calculate a local background and was subtracted from the total element signal. Background-subtracted element signals were used to calculate Cy3:Cy5 ratios. The average of the resulting total Cy3 and Cy5 signal gives a ratio that was used to balance or normalize the signals.

cDNA Synthesis. Total RNA was treated with DNase I (TaKaRa) for 10 mins at 37°C. The mixture was purified with phenol/chloroform/isoamyl alcohol. This RNA was reverse transcribed in a 40-μl reaction of 1 m*M* dNTPs, 10m*M* Tris-HCl pH8.3, 50 m*M* KCl, 5 m*M* MgCl2, 2.5 μ*M* random hexamer, 1 unit/μl RNase inhibitor, and 1 unit/μl murine leukemia virus (MuLV) reverse transcriptase

(Perkin-Elmer Corp., Foster City, CA). Reverse transcription was carried out at 42°C for 15 mins and at 99°C for 5 mins. The mixture was then cooled to 5°C and stored at -20°C until use.

RT-PCR Analysis. The quantitation of mRNA expression was carried out by the ABI Prism 7000 Sequence Detection System (Perkin-Elmer) according to the manufacturer's instructions. Oligonucleotide hybridization probes (TaqMan probe) were labeled with a 5′ fluorescent reporter dye (6FAM or VIC) and a 3′ quencher dye (TAMRA). The 5′ to 3′ nuclease activity of *Taq* DNA polymerase cleaved the probe and released the reporter, whose fluorescence was detected by the laser detector of the ABI Prism 7000. The fluorescent increase was monitored during the complete amplification. The quantitation of gene expression was derived from the cycle number at which the fluorescent signal crossed a threshold in the exponential phase of the PCR reaction by using the standard curve method according to the manufacturer's protocol. The relative intensity was normalized to an endogenous control gene (18S rRNA). The PCR amplification performed with a 20-μl final reaction mixture consisting of 10 μl 2× TaqMan Universal PCR Master mix (Perkin-Elmer), 2 μl 20X Assay mix (contained with TaqMan probe and pair primers), and 1 μl cDNA. cDNAs were amplified according to the thermal profile of 50°C for 2 mins and then 95°C for 10 mins, followed by 40 cycles at 95°C for 15 secs and 60°C for 1 min. PCR primer pairs and the TaqMan probe were designed and purchased from Perkin-Elmer. The primer and probe sequences are listed below. In all cases, the forward primer, reverse primer, and probe sequences were (i) metallothionein (MT) 2, CGCCATGGACCCCAACT, CAGGAAGTACATTTGCATTGTTTGC, and 6FAM-CCTCCGATGGATCCTG-TAMRA; (ii) S100 calcium-binding protein A9 (S100A9), AACAAAGCACCTTCT-CAGATGGA, GGTGTCCTTCCTTCCTAGAGTATTG, and 6FAM-CAGATAACCACCATCATCG-TAMRA; (iii) lipocalin 2, GCACACATCAGACCTAGTAGCTG, CAAGGCCCAGACACATGACA, and 6FAM-TCAGGGCCATGGTTTC-TAMRA; and (iv) small inducible cytokine B family member 10 (SYCB-10), TCCA-GATGCCAACCTTCCG, GGCAGCACTTGGGTTCATG, and 6FAM-AAGCCTCCCCATCAGCA-TAMRA. The sequences of 18S rRNA and MT-1, which we purchased from Perkin–Elmer, were not disclosed by the manufacturer.

Statistical Analysis. Data were reported as mean ± SEM. Differences among groups were determined by ANOVA with *post hoc* test as previously described (Stat view version 5.0; Abacus Concepts Inc., Berkeley, CA) (7).

Results

We performed cDNA microarray analysis with the LifeArray to characterize the effect of DEP on global gene expression in LPS-related acute lung injury. mRNAs were prepared from the murine lungs 4 hrs after intratracheal instillation in each group. First, we assessed the number of genes whose expression was ≥2-fold increased as compared with their expression levels in the vehicle group. Whereas 1073 genes were upregulated in the LPS group, only 38 and 204 were upregulated in the DEP and DEP + LPS groups, respectively (Table 1). However, regarding the number of the genes showing ≥6-fold increased expression, 26 genes were upregulated after combined instillation of DEP and LPS as compared with 18 genes upregulated after LPS instillation and 3 upregulated by DEP administration (Table 2). Furthermore, combined administration of DEP and LPS induced dramatic upregulation of several genes, including MT-1, MT-2, S100A9, lipocalin 2, and SYCB-10, whose gene expression levels were increased ≥20-fold in the DEP + LPS group but not in the LPS group (Table 2).

We further analyzed the gene expression of the five molecules (MT-1, MT-2, S100A9, lipocalin 2, and SYCB-10) by RT-PCR to confirm the results obtained from the cDNA microarray analysis. Combined administration of DEP and LPS prominently increased the gene expression of these molecules as compared with vehicle administration (Fig. 1). With respect to overall trends in fold increases in gene expression, the results from the RT-PCR analysis were paralleled with those from the cDNA microarray analysis.

Discussion

Our previous study has demonstrated that intratracheal instillation of DEP synergistically enhances LPS-related acute lung injury (13). This enhancement has been concomitant with the increased gene expression of IL-1β and MIP-1α in the lung and of soluble intercellular adhesion molecule 1 in the serum. In the present study, we used cDNA microarray technology to elucidate the effects of DEP on the global pattern of LPS-related gene expression in the murine lung. The results have shown that administration of DEP or LPS or both elevates the expression of various genes as compared with their expression levels induced by vehicle group (Table 1). More genes were upregulated ≥2-fold in the LPS group than in the other groups (Table 1). However, combined treatment with DEP and LPS remarkably increased the number of genes that were upregulated ≥6-fold (Table 2). In

Table 1. Number of Genes Whose Expression Was Increased ≥2-Fold as Compared with Their Expression in the Vehicle Group[a]

Group	No. of genes
DEP	38
LPS	1073
DEP + LPS	204

[a] DEP, diesel exhaust particles; LPS, lipopolysaccharide. We used a cDNA microarray to evaluate the gene expression of the four experimental groups 4 hrs after intratracheal instillation with vehicle (phosphate-buffered saline + 0.025% Tween 80), DEP (250 μg), LPS (100 μg), or DEP + LPS.

Table 2. List of Genes Whose Expression Was Increased ≥6-Fold in the Diesel Exhaust Particles (DEP) Group, the Lipopolysaccharide (LPS) Group, and the DEP + LPS Group as Compared with Their Expression in the Vehicle Group[a]

Group	Gene name	Accession number	Vehicle signal	DEP/LPS/DEP + LPS signal	Fold increase
DEP group	Metallothionein 2	W36474	283	2931	10.36
	Serum amyloid A 3	AA881525	99	671	6.78
	Metallothionein 1	AA638765	301	2036	6.76
LPS group	S100 calcium-binding protein A9 (calgranulin B)	AA255025	355	5757	16.22
	Small inducible cytokine B subfamily (Cys-X-Cys), member 1	AI158236	166	2524	15.20
	Serum amyloid A 3	AA881525	91	1267	13.92
	Lipocalin 2	AA087193	583	7744	13.28
	Metallothionein 2	W36474	351	3904	11.12
	Serine protease inhibitor 2-2	AA821980	524	5257	10.03
	Schlafen 4	AI646186	84	779	9.27
	Glycoprotein 49 B	AI894044	296	2513	8.49
	Expressed sequence AW046354	AI596632	818	6632	8.11
	Interferon-induced protein with tetratricopeptide repeats 3	AA727519	492	3770	7.66
	ESTs	AA276747	199	1471	7.39
	Metallothionein 1	AA638765	248	1831	7.38
	ESTs	AA422606	28	204	7.29
	Public domain EST	AA189760	56	397	7.09
	Small inducible cytokine A4	AA178155	120	800	6.67
	Kallikrein binding protein	W14912	385	2519	6.54
	ESTs	AI594620	31	193	6.23
	ESTs	AI426638	6485	40157	6.19
DEP + LPS group	**Lipocalin 2**	**AA087193**	**472**	**11848**	**25.10**
	S100 calcium-binding protein A9 (calgranulin B)	**AA255025**	**222**	**5421**	**24.42**
	Metallothionein 1	**AA638765**	**198**	**4736**	**23.92**
	Small inducible cytokine B subfamily (Cys-X-Cys), member 10	**AI158236**	**133**	**3005**	**22.59**
	Metallothionein 2	**W36474**	**264**	**5559**	**21.06**
	ESTs	AA276747	130	1941	14.93
	A kinase (PRKA) anchor protein (gravin) 12	AA444232	318	4376	13.76
	Serum amyloid A 3	AA881525	85	977	11.49
	Serine protease inhibitor 2-2	AA821980	384	4392	11.44
	Expressed sequence AW046354	AI596632	502	5678	11.31
	Neutrophilic granule protein	AA060282	188	1980	10.53
	Serine protease inhibitor-2 related sequence 1	AA733490	349	3292	9.43
	Glycoprotein 49 B	AI894044	208	1939	9.32
	Schlafen 4	AI646186	71	604	8.51
	Small inducible cytokine A4	AA178155	95	797	8.39
	Cholesterol 25-hydroxylase	AA289153	96	751	7.82
	S100 calcium binding protein A8 (calgranulin A)	AA458347	583	4210	7.22
	Superoxide dismutase 2, mitochondrial	AA415267	237	1673	7.06
	Intercellular adhesion molecule	W08586	812	5654	6.96
	Sema domain, immunoglobulin domain, transmembrane domain and short cytoplasmic domain, (semaphorin) 4G	AA238294	550	3682	6.69
	Expressed sequence T25557	AA511209	94	627	6.67
	Interleukin-1β	AA667815	180	1200	6.67
	T-cell specific GTPase	AI060720	317	2081	6.56
	RIKEN cDNA 3930401B19 gene	AI390138	917	5940	6.48
	Interferon-γ inducible protein, 47 kDa	AA510689	271	1660	6.13
	LPS-induced tumor necrosis factor-α	AA606770	319	1949	6.11

[a] We used a cDNA microarray to evaluate the gene expression of the four experimental groups 4 hrs after intratracheal instillation. This analysis was used on one animal for each group. The numbers of genes showing ≥6-fold increased expression as compared with the vehicle group were 26 genes in the DEP + LPS group, 18 genes in the LPS group, and 3 genes in the DEP group. Furthermore, combined administration of DEP and LPS induced dramatic upregulation of several genes, including lipocalin 2, S100 calcium-binding protein A9, small inducible cytokine B family member 10, metallothionein 1, and metallothionein 2, whose gene expression levels were increased ≥20-fold in the DEP + LPS group (bold). EST, expressed sequence Tag.

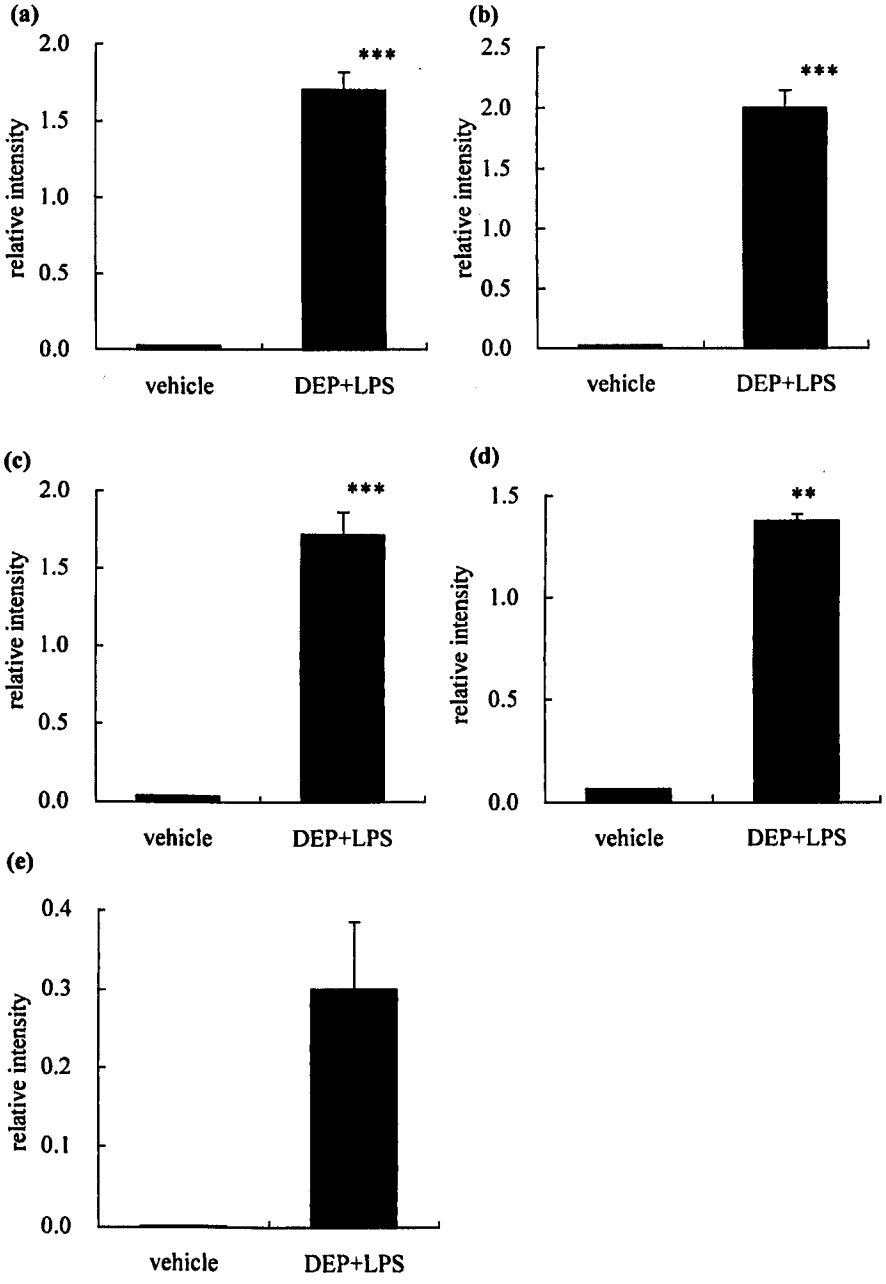

Figure 1. Reverse transcription–polymerase chain reaction analysis revealed increased gene expression of (a) metallothionein 1, (b) metallothionein 2, (c) S100 calcium-binding protein A9, (d) lipocalin 2, and (e) small inducible cytokine B family member 10, 4 hours after intratracheal administration of vehicle or diesel exhaust particles (250 μg) + lipopolysaccharide (100 μg). The relative intensity of gene expression was quantitated by the standard method according to the manufacturer's protocol and normalized to 18S rRNA. ** $P < 0.001$ versus vehicle group; *** $P < 0.0001$ versus vehicle group. Values are mean ± SEM of three animals.

particular, gene expression of MT-1, MT-2, S100A9, lipocalin 2, and SYCB-10 was ≥20-fold in the DEP + LPS group compared with their levels in the vehicle group (Table 2). The results obtained by RT-PCR analysis were consistent with those obtained by cDNA microarray analysis in the overall trend (Fig. 1).

Previous reports have suggested that DEP affect the respiratory and immune systems *in vivo* and *in vitro*. We have recently shown that DEP synergistically enhance acute lung injury related to LPS (13). The acute lung injury involved neutrophilic inflammation, lung edema, and alveolar hemorrhage. The enhancement has been concomitant with the increased expression of proinflammatory molecules, including IL-1β, MIP-1α, tumor necrosis factor-α (TNF-α), macrophage chemoattractant protein 1, keratinocyte chemoattractant, and soluble intercellular adhesion molecule 1 (13). However, our previous study has provided little information on the genes responsible for the synergistic aggravation of acute lung injury after combined exposure to DEP and LPS. In the present study, we used cDNA

microarrays to the analysis of the global gene expression pattern in the murine lung after exposure to DEP or LPS. It has been previously reported that exposure of rats to diesel exhaust induces mRNAs for A-raf and proliferating cell nuclear antigen, which are associated with pulmonary carcinogenesis (14). Koike *et al.* (15) have shown that exposure of rat alveolar macrophages to DEP extract increases the gene expression for heme oxygenase 1, heme oxygenase 2, thioredoxin peroxidase 2, glutathione S-transferase P subunit, NAD(P)H dehydrogenase, and proliferating cell nuclear antigen. Our present study showed that DEP modified the global pattern of gene expression in the presence or absence of LPS. Far more genes were activated ≥2-fold (relative to levels in the vehicle group) in the LPS group than in the other groups, but combined exposure to DEP and LPS greatly increased the number of genes that were upregulated ≥6-fold. In particular, the gene expression of MT-1, MT-2, S100A9, lipocalin 2, and SYCB-10 was increased by ≥20-fold in the DEP + LPS group but not in the LPS group (Table 2). These results indicate that combined instillation of DEP and LPS, which causes severe acute lung injury, induces more intense and specific expression of a limited number of the genes. Interestingly, the synergistic aggravation of acute lung injury by combined exposure to DEP and LPS was paralleled by the intense, focused expression of a few genes rather than the weak, broad expression of multiple genes that is induced by LPS alone.

Among the genes markedly upregulated by combined administration of DEP and LPS, S100A9 is a constitutive cytoplasmic protein in neutrophils (17). The S100A9-positive leukocytes belong to the first group of cells that invade inflammatory sites and are considered to be part of the nonspecific first line of defense against inflammatory agents. Recent reports have suggested that S100A9 is involved in the molecular processes leading to adhesion or transmigration. Extracellular S100A9 modulates the affinity of the Mac-1 integrin receptor via a G-protein–mediated mechanism (18). Furthermore, passive immunization with anti-S100A9 has inhibited the neutrophilia associated with LPS injection into the murine air pouch (19). Therefore, it is plausible that S100A9 is associated with the synergistic enhancement of acute lung injury caused by LPS and DEP.

Lipocalins are small, secreted proteins that play a role in diverse biological processes through the binding of small hydrophobic molecules and interaction with cell surface receptors (20). The lipocalin 2 sequence is 96% homologous to lipocalin 24p3 gene, a member of the lipocalin superfamily in mice. Lipocalin 24p3 binds a potent neutrophil chemoattractant (21) and possibly other lipophilic mediators of inflammation (22). Lipocalin 2 might play a similar role to lipocalin 24p3 in LPS-related inflammation. In addition, lipocalin 24p3 delivers iron to the cytoplasm, where it activates or represses iron-responsive genes (23). DEP contain a variety of heavy metals such as iron and copper (24) and generate hydroxyl radicals in the murine lungs through an iron-catalyzed reaction of superoxide and H_2O_2 (25). The synergistic aggravation of acute lung injury caused by DEP and LPS might, at least in part, be related to the iron in DEP and to the expression of lipocalin 2.

SYCB-10 is an interferon (IFN)-γ–inducible protein and a member of a small group of T-cell chemoattractants that activate the CXCR3 receptor (26). In our previous study, the DEP + LPS group showed an increase in the protein level of INF-γ in the lung tissue as compared with the vehicle group (data not shown). The functions of SYCB-10 have not been clarified, but it may play a role in the aggravation of acute lung injury in our model.

Metallothioneins are low–molecular-weight, cysteine-rich, heavy metal-binding proteins that are easily induced by heavy metals, hormones, acute stress, and various chemicals (27). MT-1 and MT-2 are expressed in all tissues and play important roles in zinc and copper homeostasis, detoxification of heavy metals, and scavenging of free radicals (28). DEP consist of carbonaceous nuclei, a variety of organic chemicals that generate free radicals (16, 29–32), and heavy metals such as iron and copper (24). On the other hand, exposure of rats to LPS has elevated the gene expression of metallothionein in the lung (33). Expression of the MT-1 and MT-2 genes might play a role in the pulmonary defense against the heavy metals in DEP or the oxidative stress caused by DEP as well as against LPS.

Our present study showed that combined administration of DEP and LPS markedly elevated the expression of molecules related to neutrophilic inflammation, such as serum amyloid A3, neutrophilic granule protein, small inducible cytokine A4, intercellular adhesion molecule, IL-1β, and LPS-induced TNF-α (Table 2). The expression levels of these genes were higher in the DEP + LPS group than in the other groups. In our previous study, the protein expression of IL-1β and soluble intercellular adhesion molecule-1 was paralleled by the mRNA expression of these molecules (13). Future studies should further examine the correlation between mRNA and protein expressions of a variety of molecules by using protein-chip technology.

In conclusion, DEP elevated the expression of a variety of genes in the murine lung in the presence or absence of LPS. In particular, combined exposure to DEP and LPS markedly increased the gene expression for MT-1, MT-2, S100A9, lipocalin 2, and SYCB-10, which showed increases ≥20-fold as compared with their expression levels after vehicle exposure. The expression of these molecules might be related to acute lung injury caused by combined exposure to DEP and LPS. The results obtained by cDNA microarray analysis were consistent with those from RT-PCR analysis in the overall trend. cDNA microarray analysis may be effective for elucidating the genes involved in biological pathways associated with DEP-related lung inflammation.

The authors thank Miho Sakurai for technical assistance.

1. Dockery DW, Pope CA III, Xu X, Spengler JD, Ware JH, Fay ME, Ferris BG Jr, Speizer FE. An association between air pollution and mortality in six U.S. cities. N Engl J Med 329:1753–1759, 1993.
2. Schwartz J. Air pollution and daily mortality: a review and meta analysis. Environ Res 64:36–52, 1994.
3. Nordenhall C, Pourazar J, Ledin MC, Levin JO, Sandstrom T, Adelroth E. Diesel exhaust enhances airway responsiveness in asthmatic subjects. Eur Respir J 17:909–915, 2001.
4. Ichinose T, Furuyama A, Sagai M. Biological effects of diesel exhaust particles (DEP). II. Acute toxicity of DEP introduced into lung by intratracheal instillation. Toxicology 99:153–167, 1995.
5. Stayner L, Dankovic D, Smith R, Steenland K. Predicted lung cancer risk among miners exposed to diesel exhaust particles. Am J Ind Med 34:207–219, 1998.
6. Ichinose T, Takano H, Miyabara Y, Yanagisawa R, Sagai M. Murine strain differences in allergic airway inflammation and immunoglobulin production by a combination of antigen and diesel exhaust particles. Toxicology 122:183–192, 1997.
7. Takano H, Yoshikawa T, Ichinose T, Miyabara Y, Imaoka K, Sagai M. Diesel exhaust particles enhance antigen-induced airway inflammation and local cytokine expression in mice. Am J Respir Crit Care Med 156:36–42, 1997.
8. Tulic MK, Wale JL, Holt PG, Sly PD. Modification of the inflammatory response to allergen challenge after exposure to bacterial lipopolysaccharide. Am J Respir Cell Mol Biol 22:604–612, 2000.
9. Wu Y, Singer M, Thouron F, Alaoui-El-Azher M, Touqui L. Effect of surfactant on pulmonary expression of type IIA PLA(2) in an animal model of acute lung injury. Am J Physiol Lung Cell Mol Physiol 282:L743–L750, 2002.
10. Kalyanaraman M, Heidemann SM, Sarnaik AP. Macrophage inflammatory protein-2 predicts acute lung injury in endotoxemia. J Invest Med 46:275–278, 1998.
11. Johnston CJ, Finkelstein JN, Gelein R, Oberdorster G. Pulmonary cytokine and chemokine mRNA levels after inhalation of lipopolysaccharide in C57BL/6 mice. Toxicol Sci 46:300–307, 1998.
12. Pacheco KA, Tarkowski M, Sterritt C, Negri J, Rosenwasser LJ, Borish L. The influence of diesel exhaust particles on mononuclear phagocytic cell-derived cytokines: IL-10, TGF-beta and IL-1 beta. Clin Exp Immunol 126:374–383, 2001.
13. Takano H, Yanagisawa R, Ichinose T, Sadakane K, Yoshino S, Yoshikawa T, Morita M. Diesel exhaust particles enhance lung injury related to bacterial endotoxin through expression of proinflammatory cytokines, chemokines, and intercellular adhesion molecule-1. Am J Respir Crit Care Med 165:1329–1335, 2002.
14. Sato H, Sagai M, Suzuki KT, Aoki Y. Identification, by cDNA microarray, of A-raf and proliferating cell nuclear antigen as genes induced in rat lung by exposure to diesel exhaust. Res Commun Mol Pathol Pharmacol 105:77–86, 1999.
15. Koike E, Hirano S, Shimojo N, Kobayashi T. cDNA microarray analysis of gene expression in rat alveolar macrophages in response to organic extract of diesel exhaust particles. Toxicol Sci 67:241–246, 2002.
16. Sagai M, Furuyama A, Ichinose T. Biological effects of diesel exhaust particles (DEP). III. Pathogenesis of asthma like symptoms in mice. Free Radic Biol Med 21:199–209, 1996.
17. Nacken W, Sopalla C, Propper C, Sorg C, Kerkhoff C. Biochemical characterization of the murine S100A9 (MRP14) protein suggests that it is functionally equivalent to its human counterpart despite its low degree of sequence homology. Eur J Biochem 267:560–565, 2000.
18. Newton RA, Hogg N. The human S100 protein MRP-14 is a novel activator of the beta 2 integrin Mac-1 on neutrophils. J Immunol 160:1427–1435, 1998.
19. Vandal K, Rouleau P, Boivin A, Ryckman C, Talbot M, Tessier PA. Blockade of S100A8 and S100A9 suppresses neutrophil migration in response to lipopolysaccharide. J Immunol 171:2602–2609, 2003.
20. Flower DR. The lipocalin protein family: structure and function. Biochem J 318:1–14, 1996.
21. Kjeldsen L, Johnsen AH, Sengelov H, Borregaard N. Isolation and primary structure of NGAL, a novel protein associated with human neutrophil gelatinase. J Biol Chem 268:10425–10432, 1993.
22. Sengelov H, Boulay F, Kjeldsen L, Borregaard N. Subcellular localization and translocation of the receptor for N-formylmethionyl-leucyl-phenylalanine in human neutrophils. Biochem J 299:473–479, 1994.
23. Yang J, Goetz D, Li JY, Wang W, Mori K, Setlik D, Du T, Erdjument-Bromage H, Tempst P, Strong R, Barasch J. An iron delivery pathway mediated by a lipocalin. Mol Cell 10:1045–1056, 2002.
24. Vouk VB, Piver WT. Metallic elements in fossil fuel combustion products: amounts and form of emissions and evaluation of carcinogenicity and mutagenicity. Environ Health Perspect 47:201–225, 1983.
25. Han JY, Takeshita K, Utsumi H. Noninvasive detection of hydroxyl radical generation in lung by diesel exhaust particles. Free Radic Biol Med 30:516–525, 2001.
26. Zlotnik A, Yoshie O. Chemokines: a new classification system and their role in immunity. Immunity 12:121–127, 2000.
27. Kägi JHR. Evolution, structure and chemical activity of class I metallothioneins. In: Metallothionein III. Biological Roles and Medical Implications; Berlin: Birkhausen Verlag, pp29–56, 1993.
28. Bremner I, Beattie JH. Metallothionein and the trace minerals. Annu Rev Nutr 10:63–83, 1990.
29. Schuetzle D. Sampling of vehicle emissions for chemical analysis and biological testing. Environ Health Perspect 47:65–80, 1983.
30. Kanoh T, Suzuki T, Ishimori M, Ikeda S, Ohasawa M, Ohkuni H, Tunetoshi Y. Adjuvant activities of pyrene, anthracene, fluoranthene and benzo(a)pyrene in production of anti-IgE antibody to Japanese cedar pollen allergen in mice. J Clin Lab Immunol 48:133–147, 1996.
31. Sagai M, Saito H, Ichinose T, Kodama M, Mori Y. Biological effects of diesel exhaust particles. I. In vitro production of superoxide and in vivo toxicity in mouse. Free Radic Biol Med 14:37–47, 1993.
32. Kumagai Y, Arimoto T, Shinyashiki M, Shimojo N, Nakai Y, Yoshikawa T, Sagai M. Generation of reactive oxygen species during interaction of diesel exhaust particle components with NADPH-cytochrome P450 reductase and involvement of the bioactivation in the DNA damage. Free Radic Biol Med 22:479–487, 1997.
33. Hur T, Squibb K, Cosma G, Horowitz S, Piedboeuf B, Bowser D, Gordon T. Induction of metallothionein and heme oxygenase in rats after inhalation of endotoxin. J Toxicol Environ Health A 56:183–203, 1999.