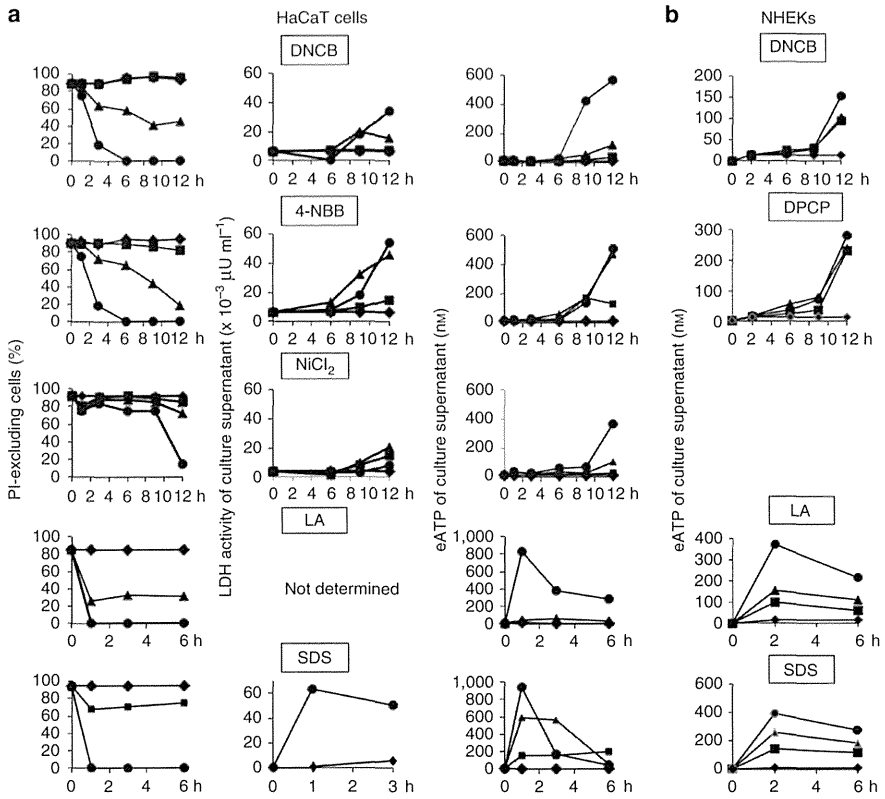


**Figure 1. Haptens and irritants cause cell death of HaCaT cells and induce adenosine 5′-triphosphate (ATP) release with different time courses.** (**a**) HaCaT cells or (**b**) normal human epidermal keratinocytes (NHEKs) cultured in 24-well plates were treated with graded concentrations of haptens dinitrochlorobenzene (DNCB), 4-nitrobenzylbromide (4-NBB), diphenylcyclopropenone (DPCP), or $NiCl_2$, or irritants lactic acid (LA) or SDS, for various time periods. After incubation, propidium iodide (PI) exclusion, lactate dehydrogenase (LDH) activity, and ATP release were examined to assess cell viability. The mean LDH activity and extracellular ATP (eATP) release of triplicate cultures were calculated for each chemical. The symbols ●, ▲, ■, and ◆ correspond to the highest, medium, and lowest concentrations and vehicle control of each chemical, respectively. Chemicals and their concentrations were as follows: DNCB, 4-NBB, and DPCP—100, 50, and 25 μM; $NiCl_2$—6.0, 3.0, and 1.5 mM; SDS—250, 125, and 62.5 μM; and LA—34 and 17 mM. *Representative data from three independent experiments are* shown.

Next, to clarify the source of ROS in HaCaT cells treated with haptens, we examined whether haptens induce mitochondrial superoxide anion generation using MitoSOX, a mitochondria-targeted ROS-specific fluorescent probe. DNCB, DPCP, and 4-NBB, although weakly, induced mitochondrial superoxide anion production 2 hours after cell exposure (Figure 3c), suggesting that mitochondria contribute to ROS production in HaCaT cells exposed to nonmetal haptens. Treatment with $H_2O_2$ also induced mitochondrial superoxide anion production. Pretreatment with NAC significantly attenuated mitochondrial superoxide anion production in HaCaT cells exposed to DNCB, 4-NBB, or DPCP, although its inhibitory effect on superoxide anion production by HaCaT cells exposed to 4-NBB was minimum (Figure 3c).

### TEMPOL and apocynin do not rescue hapten-treated HaCaT cells from cell death but suppress ROS production and reduce ATP release

Although ROS production after hapten stimulation in the mitochondria of dendritic cells (Migdal *et al.*, 2010) and in the cytosol of keratinocytes (Mehrotra *et al.*, 2005) has been documented, the source of ROS production that causes keratinocyte cell death and ATP release has not yet been determined. Therefore, we examined whether the following reagents affect ROS production in hapten-exposed HaCaT cells: TEMPOL, a whole-cell antioxidant (Wilcox and Pearlman, 2008); MnTBAP, a superoxide dismutase mimetic, catalase mimetic, and peroxynitrite scavenger (Konorev *et al.*, 2002; Batinic-Haberle *et al.*, 2009); allopurinol, a xanthine oxidase inhibitor (Borges *et al.*, 2002); and apocynin, an NADPH oxidase inhibitor (Bedard and Krause, 2007). Results showed that TEMPOL, MnTBAP, and apocynin suppressed ROS production by HaCaT cells exposed to DNCB, 4-NBB, and DPCP, but their inhibitory effect on ROS production by 4-NBB-treated HaCaT cells was not statistically significant (Figure 4a). In contrast, allopurinol failed to suppress ROS production in HaCaT cells exposed to haptens.

We also examined whether MnTBAP or TEMPOL suppresses mitochondrial superoxide anion production by

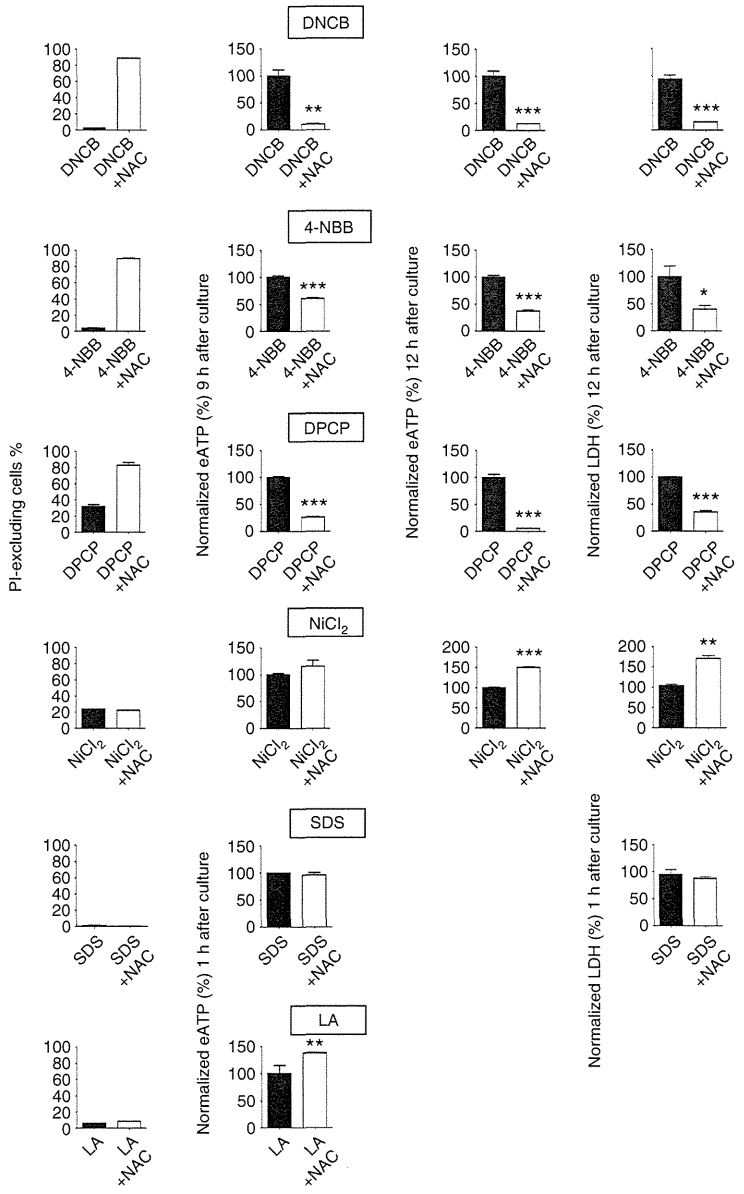


**Figure 2. *N*-acetylcysteine (NAC) significantly attenuates cell death and adenosine 5′-triphosphate (ATP) and lactate dehydrogenase (LDH) release by HaCaT cells exposed to dinitrochlorobenzene (DNCB), 4-nitrobenzylbromide (4-NBB), or diphenylcyclopropenone (DPCP) but does not affect the death or ATP release of cells exposed to $NiCl_2$, SDS, or lactic acid (LA).** HaCaT cells cultured in 24-well plates were either pretreated or not with 2.5 mM of NAC for 30 minutes, followed by exposure to 100 μM of DNCB, 4-NBB, or DPCP, 6 mM of $NiCl_2$, 250 μM of SDS, or 34 μM of LA for various time periods. The effects of NAC on cell death, extracellular ATP (eATP) levels, and LDH activity were assessed 6 hours after culture by propidium iodide (PI) exclusion assay, 9 and 12 hours after culture, and 12 hours after culture, respectively. The mean eATP or LDH activity of triplicate cultures was calculated for each chemical, and results were normalized to the data of hapten-exposed HaCaT cells without NAC. Bars represent mean ± SD. Significant differences between treatment groups: $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$. Representative data from three independent experiments are shown.

hapten-exposed HaCaT cells. In contrast to its effects on ROS production in HaCaT cells, MnTBAP did not suppress mitochondrial superoxide anion production (Figure 4b). TEMPOL suppressed the MitoSOX fluorescence intensity of HaCaT cells exposed to 4-NBB, whereas it did not affect the fluorescence of cells exposed to DNCB or DPCP (Figure 4b).

Next, we examined whether ROS is involved in cell death or ATP release in HaCaT cells exposed to haptens. Assessment

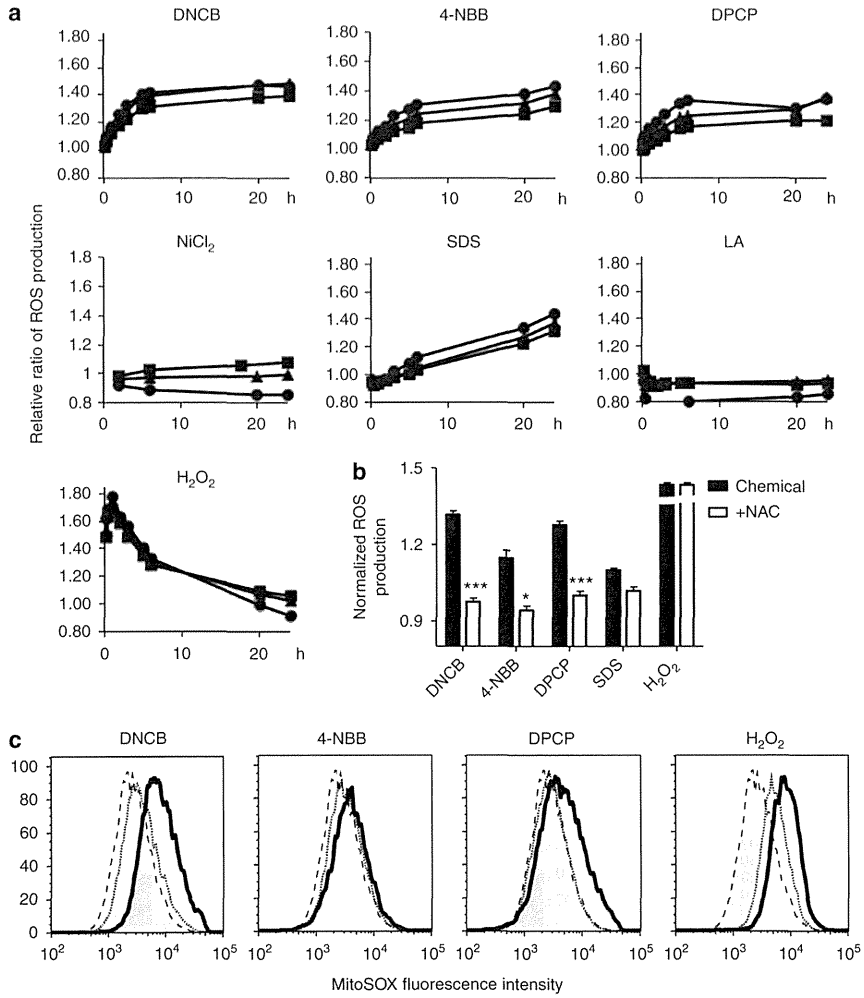


**Figure 3. HaCaT cells treated with haptens generate reactive oxygen species (ROS) and mitochondrial superoxide anion in a dose-dependent manner that are significantly reduced by *N*-acetylcysteine (NAC).** (**a**) HaCaT cells were treated with graded concentrations of haptens, irritants, or $H_2O_2$ as a positive control for various time periods. After culture, intracellular ROS were measured using a CM-$H_2$DCFDA probe. The mean fluorescence intensity of triplicate cultures was calculated for each chemical and expressed as percentage change. The symbols ■, ▲, and ● correspond to the lowest, medium, and highest concentrations, respectively. Chemicals used and their concentrations were as follows: dinitrochlorobenzene (DNCB), 4-nitrobenzylbromide (4-NBB), and diphenylcyclopropenone (DPCP)—25, 50, and 100 μM; $NiCl_2$—1.5, 3, and 6 mM; SDS—62.5, 125, and 250 μM; lactic acid (LA)—17 and 34 mM; and $H_2O_2$—44, 88, and 176 μM. (**b**) HaCaT cells either pretreated or not with NAC were exposed to haptens, SDS, or $H_2O_2$ for 6 hours. After culture, intracellular ROS were measured with a CM-$H_2$DCFDA probe. The mean fluorescence intensity of triplicate cultures was calculated for each chemical, and the data were normalized to the intensity of nontreated HaCaT cells. Bars represent mean ± SD. Significant differences between treatment groups: $^*P<0.05$, $^{***}P<0.001$. Representative data from three independent experiments are shown. (**c**) MitoSOX-preloaded HaCaT cells were either treated or not treated with NAC, followed by exposure to haptens or $H_2O_2$. The MitoSOX fluorescence was measured using a flow cytometer. The solid, dotted, and ruptured lines and the shaded area of the histograms represent HaCaT cells treated with hapten alone, hapten + NAC, vehicle control + NAC, and vehicle control alone, respectively.

of cell death by the PI exclusion assay showed that TEMPOL, MnTBAP, apocynin, and allopurinol could not rescue HaCaT cells from cell death after hapten treatment (Supplementary Figure S1 online). TEMPOL, MnTBAP, and apocynin reduced LDH activity and ATP release from DNCB- and 4-NBB-exposed HaCaT cells (Figure 4c and d), and TEMPOL attenuated LDH activity and ATP release from DPCP-exposed HaCaT cells (Supplementary Figure S2 online).

### Inhibition of Panx-1 channels significantly suppresses ATP release from hapten-treated HaCaT cells

Multiple pathways other than cell lysis are involved in ATP release (Lohman *et al.*, 2012). It has been demonstrated that ATP release into the extracellular space by dying cells during apoptosis depends on the Panx channel (Chekeni *et al.*, 2010). The release of ATP through Panx hemichannels has also been reported in the setting of ischemia-induced oxidative stress

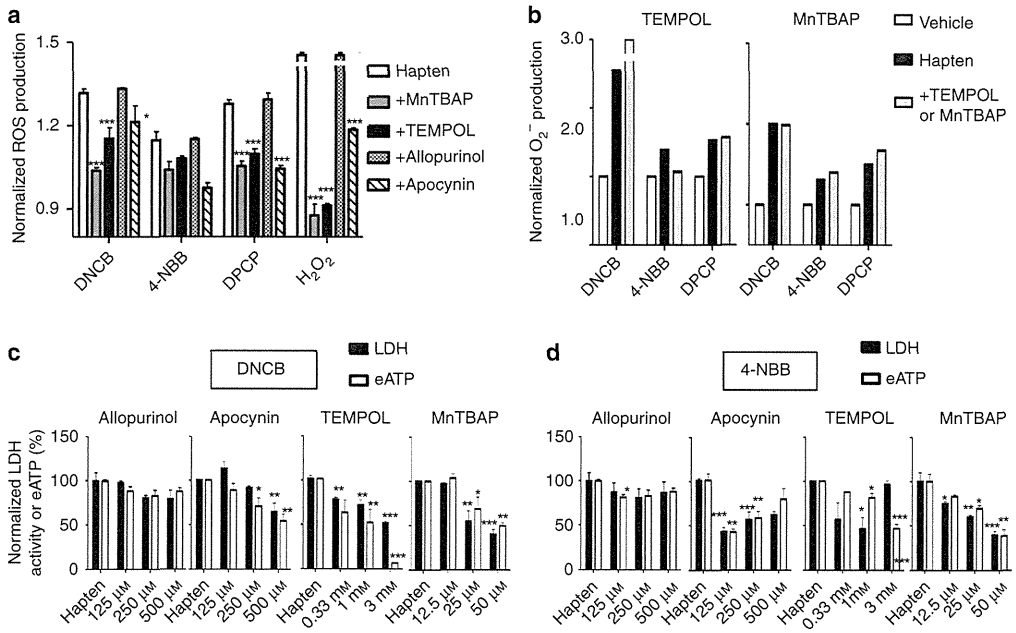


**Figure 4. Antioxidants significantly suppress reactive oxygen species (ROS) production by hapten-treated HaCaT cells, and reduce lactate dehydrogenase (LDH) activity and adenosine 5′-triphosphate (ATP) release without decreasing the number of propidium iodide (PI)-positive cells.** (**a**) HaCaT cells were either pretreated or not with antioxidants for 30 minutes, followed by exposure to haptens or $H_2O_2$. At 6 hours after culture, intracellular ROS were measured using a CM-$H_2$DCFDA probe. (**b**) MitoSOX-preloaded HaCaT cells were pretreated with TEMPOL or MnTBAP for 30 minutes, followed by exposure to haptens for 2 hours. The MitoSOX fluorescence was measured using a flow cytometer. Mean fluorescence intensity was calculated for each chemical, and the data were normalized to the intensity of nontreated HaCaT cells. (**c**, **d**) HaCaT cells were either pretreated or not with antioxidants for 30 minutes, followed by exposure to haptens. At 12 hours after culture, the LDH activity and ATP release in the culture supernatants were measured by colorimetric and luciferase assays, respectively. The mean LDH activity and ATP release of triplicate cultures were calculated for each chemical, and results were normalized to the data of hapten-treated HaCaT cells without inhibitors. Bars represent mean ± SD. Significant differences between treatment groups: $*P<0.05$, $**P<0.01$, $***P<0.001$.

culminating in the cell death of oligodendrocytes (Domercq *et al.*, 2010). We therefore examined whether CBX, a nonspecific Panx inhibitor (Suadicani *et al.*, 2006; Ma *et al.*, 2009), can suppress ATP release from hapten-treated HaCaT cells (Figure 5a). Interestingly, CBX significantly decreased ATP release from hapten-treated but not irritant-treated HaCaT cells in a dose-dependent manner. Furthermore, CBX suppressed LDH release from DNCB-treated but not 4-NBB- or DPCP-treated HaCaT cells (data not shown). Evaluation of cell death by the PI exclusion assay showed that CBX was unable to rescue hapten-exposed HaCaT cells from cell death.

To exclude the possibility that CBX reduced levels of intracellular ATP, thereby decreasing ATP release from hapten-treated cells, we examined the concentration of intracellular ATP in DNCB-exposed HaCaT cells. Results showed that CBX at concentrations of 7.8 to 31 μM increased intracellular ATP levels and decreased ATP release, whereas CBX at 62 μM slightly decreased the intracellular ATP level and significantly decreased ATP release (Figure 5b). These findings exclude the possibility that CBX reduces ATP release by depleting intracellular ATP.

To further examine the role of Panx hemichannels in ATP release from hapten-treated HaCaT cells, we examined the effects of another Panx-1 inhibitor, probenecid (Silverman *et al.*, 2008), and a Panx-1 mimetic blocking peptide (Pelegrin and Surprenant, 2006) on ATP release from hapten-treated HaCaT cells. Probenecid significantly suppressed ATP release from DNCB- or 4-NBB-treated HaCaT cells, and Panx1-blocking peptide also significantly inhibited ATP release from DNCB-treated HaCaT cells (Figure 5c and d). We also examined the effect of small interfering RNA (siRNA) against Panx1. Attenuation of Panx1 mRNA expression in HaCaT cells by Panx1 siRNA significantly suppressed ATP release from cells exposed to either DNCB or DPCP (Figure 5e and f).

### Inhibition of Panx1 by CBX significantly reduces CHS induced by DNCB

Finally, to explore the role of Panx1 in the induction of CHS, we administered CBX by intraperitoneal injection and induced CHS using DNCB. After challenge with 0.5% DNCB, the ear swelling of mice pretreated with CBX was significantly reduced compared with those of saline-injected control mice, suggesting that CBX attenuated the CHS response (Figure 6). In contrast, CBX treatment did not affect the ear swelling induced by 0.5% DNCB without sensitization.

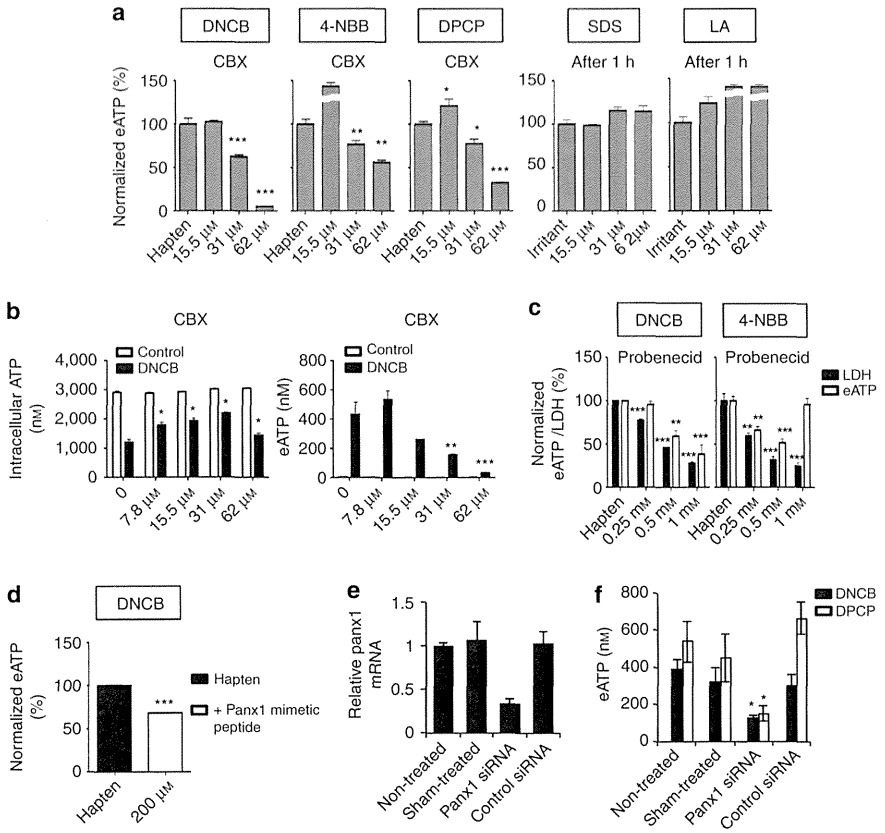


**Figure 5. Carbenoxolone (CBX), probenecid, and a pannexin-1 (Panx1) mimetic blocking peptide suppress adenosine 5′-triphosphate (ATP) release from hapten-treated HaCaT cells.** (**a**) HaCaT cells were either pretreated or not pretreated with graded concentrations of CBX, followed by exposure to haptens for 12 hours. After culture, ATP in the culture supernatants was measured by luciferase assay. (**b**) The intracellular ATP of HaCaT cells and ATP in the culture supernatants from the same culture were measured by luciferase assay. HaCaT cells were pretreated with (**c**) probenecid or a (**d**) Panx1 mimetic blocking peptide for 30 minutes, followed by exposure to haptens for 12 hours. After culture, ATP or lactate dehydrogenase (LDH) activity in the culture supernatants was measured. The mean extracellular ATP (eATP) and LDH activity of triplicate cultures was calculated for each chemical and results were normalized to the data of hapten-exposed HaCaT cells without inhibitors. Bars represent mean ± SD. Significant differences between treatment groups: $*P<0.05$, $**P<0.01$, $***P<0.001$. HaCaT cells were either treated or not with 10 nM of Panx1 siRNA or control siRNA in transfection reagent. (**e**) After 51 hours of culture, Panx1 mRNA expression in each treatment group was measured by quantitative real-time PCR. (**f**) Cells were subsequently treated with dinitrochlorobenzene (DNCB) or diphenylcyclopropenone (DPCP) for 12 hours and the recovered supernatants were used to measure eATP. Significant differences between treatment groups: $*P<0.05$.

## DISCUSSION

In this study, we demonstrated that the haptens DNCB, 4-NBB, DPCP, and $NiCl_2$, and the irritants killed keratinocytes and induced ATP release from keratinocytes with different time courses. This suggests that the mechanism of hapten-induced keratinocyte cell death leading to ATP release is different from that of irritants. Furthermore, keratinocyte cell death caused by nonmetal haptens DNCB, 4-NBB, and DPCP, but not cell death caused by the metal hapten $NiCl_2$ or by irritants, was abrogated by NAC. The fact that NAC is a thiol-containing compound that interferes with thiol redox transitions (Parasassi *et al.*, 2010), and that haptens exhibit a strong affinity toward thiol groups (Becker *et al.*, 2003), suggests that nonmetal haptens kill keratinocytes via reactivity to thiol residues in keratinocytes. In contrast, the mechanism of cell death induced by $NiCl_2$ or irritants was not dependent on this.

Next, we demonstrated that only nonmetal haptens induced ROS production by HaCaT cells that was significantly attenuated by NAC treatment. Again, this suggests that thiol modification by haptens has a crucial role in ROS production. Apart from NAC, MnTBAP, TEMPOL, and apocynin significantly suppressed ROS production by hapten-treated HaCaT cells. However, the three antioxidants did not decrease cell death as evaluated by PI exclusion that suggests that ROS generated by hapten-treated HaCaT cells does not cause membrane disruption.

On the other hand, the three antioxidants suppressed ATP and LDH release from hapten-treated HaCaT cells. We found that ATP and LDH release from hapten-treated HaCaT cells

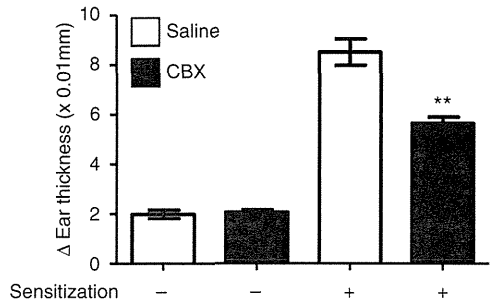


**Figure 6. Inhibition of pannexin-1 (Panx1) by carbenoxolone (CBX) significantly reduces contact hypersensitivity (CHS) induced by dinitrochlorobenzene (DNCB).** To examine the role of extracellular adenosine 5′-triphosphate (eATP) in sensitization, C57Bl/6 mice were given an intraperitoneal injection of CBX or saline on day 0 and then sensitized with 1% DNCB (or without sensitization) on days 0, 1, and 2, followed by epicutaneous application of 20 μl of 0.5% DNCB on the dorsum of both ears on day 4. Ear measurement was taken on days 4 and 6. The data represent the mean increase in ear thickness for groups of seven mice ± SD. Representative data from three independent experiments are shown. $^{**}P<0.01$ (vs. saline control, Student's *t*-test).

was evident 6 hours after hapten treatment, whereas they started to incorporate PI from 1 hour after exposure. As the molecular weights of LDH and PI are ~140,000 and 688 Da, respectively, we speculate that considerable time is required for sufficient membrane disruption to occur that permits the passage of large molecules. However, molecular size alone cannot fully explain the delay in ATP release as the molecular weight of ATP is lower than that of PI.

It has been reported that ischemia-related oxidative stress culminating in the cell death of oligodendrocytes induced ATP release through the opening of Panx hemichannels (Domercq *et al.*, 2010). As Panx1 is ubiquitously expressed in human tissues including the skin (Baranova *et al.*, 2004), we hypothesized that ROS produced by hapten-treated HaCaT cells may open Panx hemichannels. Indeed, Panx inhibitors as well as Panx1 siRNA significantly attenuated ATP release from HaCaT cells exposed to DNCB, 4-NBB, or DPCP. In addition, significant suppression of ATP release by antioxidants in a dose-dependent manner suggests that ROS production by hapten-treated HaCaT cells has a role in the opening of Panx hemichannels. Combined, our findings suggest that irritants induce ATP release from keratinocytes by disrupting cell membranes, whereas nonmetal haptens such as DNCB, 4-NBB, and DPCP induce ATP release from keratinocytes via ROS-mediated opening of Panx1 channels. Therefore, it is conceivable that Panx hemichannels have a crucial role in sensitization, just as eATP and $P2X_7$ are essential in the mouse CHS model (Weber *et al.*, 2010). This is supported by observation of the attenuated CHS response after CBX pretreatment in mice.

This study also demonstrated differences in the mechanism of ATP release among haptens. ATP release from HaCaT cells treated with the metal hapten $NiCl_2$ was independent of thiol reactivity of $NiCl_2$, whereas nonmetal hapten-induced ATP release from HaCaT cells was dependent on reactivity to thiol residues and ROS production. It has been reported that Ni can stimulate human TLR4 (Schmidt *et al.*, 2010) that suggests that Ni utilizes the TLR4 pathway to activate the innate immune response instead of generating ROS in allergic sensitization. In addition, there was quantitative difference in ROS and superoxide anion production among nonmetal haptens. It is conceivable that different nonmetal haptens generate ROS by different mechanisms dependent on their own chemical properties. Further studies are required to examine the precise mechanism by which nonmetal haptens generate ROS and/or superoxide anion and open Panx channels.

In this study, we attempted to determine the source of ROS in keratinocytes after hapten exposure. Although superoxide anion production by mitochondria occurred after hapten exposure, antioxidants such as TEMPOL and MnTBAP did not attenuate mitochondrial superoxide anion production despite their suppression of ROS production, LDH activity, and ATP release. This suggests that ROS production by mitochondria does not have a significant role in ATP release from hapten-treated keratinocytes, consistent with the observation by Mehrotra *et al.* (2005). However, our study could not determine which cytosolic compartment or enzyme was responsible for ROS production that led to the release of ATP. Although Kim *et al.* (2012) and Esser *et al.* (2012) demonstrated ROS production and mitochondrial superoxide anion production by hapten-treated keratinocytes, neither group succeeded in identifying the source of ROS production that influenced IL-1α production, ICAM-1 expression, or induction of hyaluronidase activity.

Our study provides an insight into the mechanism by which haptens kill keratinocytes and cause a large release of ATP. These findings provide additional evidence of the crucial role of keratinocytes in the sensitization of CHS. In addition, the results of this study suggest that Panx1 may be targeted to protect humans from sensitization by haptens. The Panx1 inhibitor CBX has already been approved as a cosmetic ingredient and may be useful as a topical agent in inflammatory or immune skin diseases by modulating innate immunity.

## MATERIALS AND METHODS

### Test chemicals and preparation of chemicals

Four contact sensitizers (DNCB, 4-NBB, $NiCl_2$, and DPCP) and two irritants (SDS and LA) were used. The following antioxidants were used in experiments: NAC, allopurinol, MnTBAP, and apocynin. Panx was inhibited using carbenoxolone disodium salt (CBX), probenecid, or Panx-1 mimetic blocking peptide. Full details are available in the Supplementary Methods online.

### Keratinocyte culture

HaCaT cells, a gift from Norbert Fusenig in Heidelberg, Germany, and neonatal foreskin NHEKs purchased from Kurabo (Osaka, Japan) were used in this study. Full details regarding cell culture are available in the Supplementary Methods online.

### Chemicals exposure of keratinocytes

HaCaT cells or NHEKs were cultured in 24-well plates, washed twice 48 hours later, and incubated with DMEM without phenol red at

37 °C in 10% $CO_2$ for 1 hour. Afterwards, they were pretreated with or without graded concentrations of antioxidants or Panx inhibitors for 30 minutes, followed by treatment with graded concentrations of haptens or irritants for various time periods at 37 °C in 10% $CO_2$.

### Knockdown of Panx1 by stealth siRNA

In some experiments, HaCaT cells were treated with siRNA against Panx1 as described previously (Hirakawa *et al.*, 2011), followed by hapten exposure. Full details are available in the Supplementary Methods online.

### Cell viability

Cell viability was determined by either a PI exclusion assay using flow cytometry or LDH release. Full details are available in the Supplementary Methods online.

### Measurement of intracellular ROS

Intracellular ROS were measured fluorometrically using a CM-$H_2$DCFDA probe (Invitrogen, Grand Island, NY) according to the manufacturer's protocol. Full details are available in the Supplementary Methods online.

### *In vitro* detection of mitochondrial superoxide anion

Mitochondrial superoxide anion was detected by MitoSOX RED (Invitrogen). Full details are available in the Supplementary Methods online.

### Measurement of ATP

The extracellular ATP level was measured with a commercially available kit (ENLITEN, rLuciferase/Luciferin Reagent; Promega, Madison, WI). Full details are available in the Supplementary Methods online.

### Murine model of CHS

Female C57Bl/6 mice were sensitized by painting the shaved abdominal skin with 100 μl of 1% DNCB in 4:1 (v/v) acetone/olive oil on days 0, 1, and 2. For elicitation, 20 μl of 0.5% DNCB was applied to the dorsum of both ears on day 4. To examine the role of eATP in sensitization, we injected 20 mg $kg^{-1}$ of CBX into the peritoneum on day 0. Full details are available in the Supplementary Methods online.

### Statistical analysis

At least three independent experiments were performed for each analysis and representative data from one experiment are shown. A one-way or two-way analysis of variance test was used to evaluate statistical significance. The *P*-values of $<0.05$ were considered statistically significant.

## CONFLICT OF INTEREST

The authors state no conflict of interest.

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## SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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ORIGINAL ARTICLE

# Effects on asthma and induction of interleukin-8 caused by Asian dust particles collected in western Japan

Masanari Watanabe, MD, PhD[1], Jun Kurai, MD, PhD[1], Katsuyuki Tomita, MD, PhD[2], Hiroyuki Sano, MD, PhD[3], Satoshi Abe, PhD[4], Rumiko Saito, PhD[5], Sayaka Minato, Tadashi Igishi, MD, PhD[1], Naoto Burioka, MD, PhD[1], Takanori Sako, MD, PhD[7], Kazuhito Yasuda, MD, PhD[8], Masaaki Mikami, MD, PhD[9], Shinichi Kurita, MD, PhD[7], Hirokazu Tokuyasu, MD, PhD[10], Yasuto Ueda, MD, PhD[1], Tatsuya Konishi, MD, PhD[11], Akira Yamasaki, MD, PhD[1], Setsuya Aiba, MD, PhD[12], Mitsuo Oshimura, PhD[4], and Eiji Shimizu, MD, PhD[1]

*[1]Department of Respiratory Medicine and Rheumatology, Faculty of Medicine, Tottori University, Yonago, Tottori, [2]Department of Respiratory Medicine, Yonago Medical Center, Yonago, Tottori, [3]Department of Respiratory Medicine and Allergology, Faculty of Medicine, Kinki University, Osakasayama, Osaka, [4]Chromosome Engineering Research Center, Tottori University, Tottori, [5]Department of Integtative Genomics, Tohoku Medical Megabank Organization, Tohoku University, Sendai, Miyagi, [6]Tottori Prefectural Institute of Health and Environment, Yurihama, Tottori, [7]Department of Respiratory Medicine, Obase Hospital, Kanda, Fukuoka, [8]Yasuda Clinic, Toyooka, Hyogo, [9]Hosshoji Clinic, Saihaku, Tottori, [10]Department of Respiratory Medicine, Matsue Red Cross Hospital, Matsue, Shimane, [11]Department of Respiratory Medicine, Matsue City Hospital, Matsue, Shimane, and [12]Department of Dermatology, Graduate School of Medicine, Tohoku University, Sendai, Miyagi*

**Abstract**

*Objective*: Asian dust storms (ADS) contain various airborne particles that may augment airway inflammation by increasing the level of interleukin-8. The objective of the study was to investigate the association of exposure to an ADS with worsening of symptoms of adult asthma and the effect of ADS particles on interleukin-8 transcriptional activity. *Methods*: The subjects were 112 patients with mild to moderate asthma who recorded scores for their daily upper and lower respiratory tract symptoms and measured morning peak expiratory flow (PEF) from March to May 2011. Interleukin-8 transcriptional activity was assessed in THP-G8 cells that were exposed to airborne particles collected during days of ADS exposure. *Results*: Of the 112 patients, 31 had comorbid allergic rhinitis (AR) and/or chronic sinusitis (CS), and had worsened scores for upper respiratory tract symptoms on ADS days compared to non-ADS days. Scores for lower respiratory tract symptoms during ADS days were higher than non-ADS days in all patients. Three patients also had unscheduled hospital visits for exacerbation of asthma on ADS days. However, there was no significant difference in daily morning PEF between ADS and non-ADS days. Airborne particles collected on ADS days induced interleukin-8 transcriptional activity in THP-G8 cells compared to the original soil of the ADS. *Conclusion*: Exposure to an ADS aggravates upper and lower tract respiratory symptoms in patients with adult asthma. ADS airborne particles may increase airway inflammation through enhancement of interleukin-8 transcriptional activity.

**Keywords**

Asian dust storms, interleukin-8, luciferase assay, respiratory tract, THP-G8 cells

**History**

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## Introduction

Exposure to particulate matter is associated with respiratory and cardiovascular morbidity or mortality [1,2]. In pediatric asthma patients, Strickland et al. found that short-term exposure to air pollutants increased the number of emergency department visits, even with exposure at relatively low concentrations [3]. Desert dust, which is considered to be harmless, can increase the prevalence of asthma and the occurrence of asthma symptoms [4–6]. Asian dust storms (ADS) originating in the deserts of Mongolia, northern China, and Kazakhstan often disperse dust over East Asia from spring until late autumn. In Japan, the highest frequency of ADSs has occurred from March to May in recent years, based on information from the Japan Meteorological Agency. An ADS can occasionally be large enough to reach the west coast of the United States [7,8] and Uno et al. [9] showed that an ADS can spread around the globe.

Recent studies have shown an association of ADSs with an increased risk of exacerbation of asthma [10–13]. Our previous telephone surveys also showed that an ADS can aggravate symptoms and pulmonary dysfunction in adult patients with asthma [14,15]. However, it has been suggested that an ADS is not significantly associated with the risk of hospitalization for asthma or the incidence of asthma attacks in Taipei [16,17]. Min et al. showed that yellow sand aggravated upper respiratory tract symptoms in adult patients

Correspondence: Masanari Watanabe, M.D., Ph.D, Department of Respiratory Medicine and Rheumatology, Faculty of Medicine, Tottori University, 36-1 Nishimachi, Yonago 683-8504, Japan. Tel: +81 859386537. Fax: +81 859386539. E-mail: watanabm@med.tottori-u.ac.jp

with asthma [18], but few studies have examined the association of ADS exposure with upper respiratory tract symptoms in adult asthma.

In animal models, airborne particles originating from ADSs in Mongolia and northern China have been shown to increase pulmonary inflammation and infiltration [19–21]. The mechanism is unclear, but Sierra-Vargas et al. showed that neutrophils migrate to the lung during acute inflammation induced by exposure to air pollutants [22]. Air pollutants also increase the concentration of interleukin (IL)-8 in bronchial lavage fluid (BALF) and IL-8 mRNA expression in bronchial biopsy tissue obtained from healthy subjects [23]. IL-8 increases in the blood of asthma patients during exacerbation [24], and thus is thought to be a key cytokine in exacerbation of asthma.

In this study, we investigated the relationship between exposure to an ADS and upper and lower respiratory tract symptoms and respiratory function in adult patients with controlled asthma. An IL-8 luciferase assay in a stable THP-1-derived IL-8 reporter cell line was used to investigate the effect of airborne ADS particles.

## Materials and methods

### Patients

A total of 184 outpatients aged >18 years old with asthma were recruited into the study from December 2010 to January 2011. The patients were residents in four different locations, Yonago City, Matsue City, Toyooka City, and Kanda Town, which are distributed in a rural area of under 200 km diameter in western Japan. Of these patients, 112 were included in the analysis based on the following inclusion criteria (Figure 1): (1) mild to moderate asthma, as defined by the National Heart, Lung, and Blood Institute [25], (2) controlled asthma within 3 months before March 2011, based on the Global Initiative for Asthma (GINA) definition [26], (3) recorded daily respiratory symptoms and PEF for >90% of days from March to May, 2011, and (4) no hospitalization except for asthma from March to May, 2011. On the basis of GINA criteria, asthma was defined as positive if a case met (1) and (2) or (3) of the following criteria: (1) a history of intermittent wheezing; (2) airway hyper-responsiveness to methacholine; and (3) reversible airflow limitation (12% and 200 ml variability in $FEV_1$). The Research Ethics Committees of each participating institution approved the study and all patients gave written informed consent.

### Definition of the period of ADS exposure and monitoring of air pollutants

The period of ADS exposure was determined using information from the Japan Meteorological Agency based on a criterion of visibility <10 km due to dust arising from the deserts of East Asia, as determined by meteorological satellites. Concentrations of suspended particular matter (SPM), sulfur dioxide ($SO_2$), nitrogen dioxide ($NO_2$), and photochemical oxidants ($O_x$) were confirmed based on monitoring at many locations in Japan by the Japanese Ministry of the Environment. In this study, we used these data for concentrations of SPM, $SO_2$, $NO_2$, and $O_x$ at the four study locations.

### Recording of daily upper and lower respiratory tract symptoms and PEF

From February to May 2011, all patients recorded their daily respiratory tract symptoms and measured their morning PEF using a peak flow meter (Mini-Wright, Harlow, England, American Thoracic Society scale). February was the practice period. Scores for upper respiratory tract symptoms such as stuffiness, sneezing, and lower respiratory tract symptoms such as cough, sputum, dyspnea, and wheezing were recorded in a diary using 0 = absent, 1 = mild, and 2 = severe for each

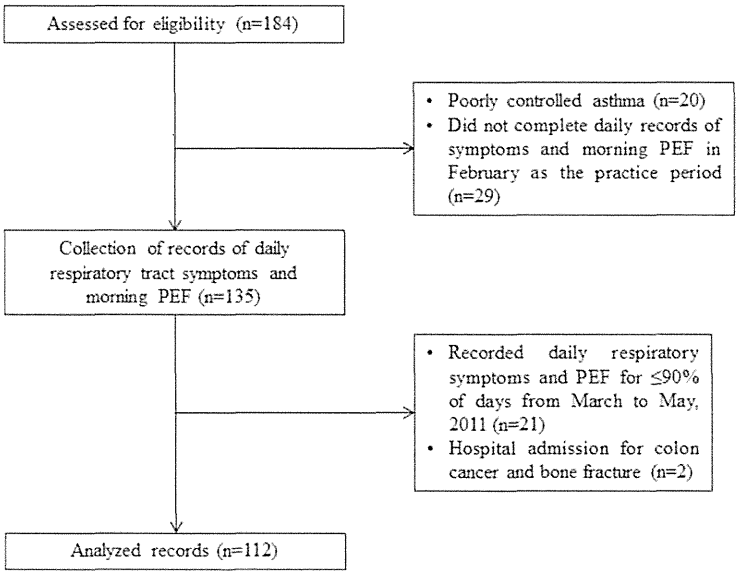


Figure 1. Flowchart showing the disposition of subjects in the study.

symptom. Unscheduled hospital visits, fever and pharyngeal pain, and use of oral corticosteroids for exacerbation of asthma on ADS days were also recorded by the patients. Respiratory tract infection was defined as being present if patients had pharyngeal pain and/or fever, which was defined as a body temperature >37.5 °C.

The total scores for each upper and lower respiratory tract symptom was calculated by summing the score for each day. The period of ADS exposure was determined to be 1–3 May 2011 using information from the Japanese Ministry of the Environment. The results were analyzed over a period of one month because climate conditions differ markedly between March and May in Japan. Thus, the control period of non-ADS exposure was defined as 10–30 April 2011. Patients with worsened symptoms associated with ADS were defined as those with wheezing or a severe score for more than two symptoms out of cough, sputum, and dyspnea without pharyngeal pain and/or fever during the ADS period. Patients recorded the best PEF value from three attempts within 30 min of waking up and before taking inhaled corticosteroids (ICS), $\beta_2$-agonists or oral drugs. The minimum morning PEF (% recent best) was determined based on the lowest daily morning prebronchodilator PEF [27]. The recent best value is defined as the best PEF from February to May 2011 in each patient. The lowest PEF values were also determined for the ADS (1 to 3 May 2011) and non-ADS (10 to 30 April 2011) periods.

### Preparation of airborne particles collected on ADS days

Soil from the China Loess Plateau (CJ-1), the original ADS soil in the Tengger Desert and Huining located in Gansu Province, was obtained from the National Institute for Environmental Studies (Ibaraki, Japan) in 2002. This reference material was certified by the National Institute for Environmental Studies and the National Research Center for Environmental Analysis and Measurement (Beijing, China). Dust particles were collected in Yonago City on ADS days (1–3 May 2011) using a large acrylic basin with a collection area of 5000 $cm^2$ and a depth of 30 cm (custom made by Denyo Inc., Tokyo, Japan). CJ-1 and the collected dust were sterilized at 121 °C for 30 min in an autoclave (Tomy SX-300; Tomy Co., Tokyo, Japan) and stored in a freezer at −20 °C to prevent growth of bacteria and fungi. For stimulation of THP-G8 cells, airborne particles collected on ADS days were diluted to various concentrations with distilled deionized water. Supernatant extracted from water-soluble airborne particles was also collected after the particles were kept on ice for 1 h.

### IL-8 promoter-luciferase gene reporter assay

THP-G8 cells are a THP-1-derived reporter cell line that express stable luciferase orange (SLO) and stable luciferase red (SLR) genes under the control of the IL-8 and glyceraldehyde 3-phosphate dehydrogenase promoters, respectively [28]. The THP-G8 cell line was kindly provided by the Department of Dermatology, Tohoku University, Graduate School of Medicine (Sendai, Japan) and was cultured according to a previous report [28]. To measure the changes of SLO and SLR luciferase activity after exposure to CJ-1 soil and ADS airborne particles, THP-G8 cells ($5 \times 10^4$ cells/100 µl/well) in 96-well black plates (Greiner Bio-One GmbH, Frickenhausen, Germany) were stimulated for 5 h with solvent only (negative control), 100 ng/ml lipopolysaccharide (LPS) (Wako Pure Chemicals, Osaka, Japan), and various concentrations of CJ-1 soil and ADS airborne particles. The IL-8 transcriptional activity of THP-G8 cells was evaluated at maximum induction after stimulation by 100 ng/ml LPS for 5 h [28]. Luciferase activity was determined using a microplate luminometer with a Phelios multicolor detection system (Atto Co., Tokyo, Japan) using Tripluc luciferase assay reagent (Toyobo Co., Osaka, Japan). IL-8 transcriptional activity was assessed from normalized SLO luciferase activity (nSLO-LA), which was calculated as SLO-LA divided by SLR-LA, and the fold induction of nSLO-LA was calculated as the nSLO-LA level of treated cells divided by that of untreated cells [28].

### Measurement of concentrations of IL-8 and endotoxin

IL-8 concentrations in culture supernatants were determined using an enzyme-linked immunosorbent assay (ELISA) kit for IL-8 (R&D Systems, Minneapolis, MN, USA). Samples were run in triplicate and read using an automated ELISA reader (Bio-Rad Model 680, Bio-Rad, Philadelphia, PA). The range of the assay was 31.2 to 2000 pg/ml. The endotoxin concentration in ADS airborne particles was measured with a Chromogenic LAL endotoxin assay kit (GenScript, Piscataway, NJ, USA). The range of the assay was 0.01 to 1 EU/ml. The pH of ADS airborne particles was measured with a pH meter (MP220; Mettler Toledo, Schwerzenbach, Switzerland).

### Statistical analysis

Results are shown as the mean ± standard deviation (SD). SPSS Statistics software (Japanese ver. 16.0 for Windows; IBM Japan, Tokyo, Japan) was used for statistical analysis. A Mann–Whitney U test was used for comparison of air pollution data between the ADS and non-ADS periods. A $\chi^2$ test was used for comparison of categorical data between patients with and without comorbid allergic rhinitis and chronic sinusitis. Pulmonary function in these two groups was analyzed by Mann–Whitney *U* test. Comparisons of the average score for daily upper and lower respiratory tract symptoms between the ADS and non-ADS periods were analyzed by Wilcoxon signed rank test. The difference in average PEF in the ADS and non-ADS periods was analyzed by *t*-test. Significance was defined as $p < 0.05$ in all analyses.

## Results

### Patient characteristics

A flowchart showing the progression of subjects through the study is shown in Figure 1. Of the initial 184 outpatients with asthma, 20 were excluded due to poorly controlled asthma and 29 declined to record daily respiratory symptoms and morning PEF during the practice period in February. Of the

remaining 135 patients, 21 did not record daily respiratory symptoms and morning PEF for >90% of days from March to May and 2 were excluded due to hospital admissions for colon cancer and bone fracture. This left 112 patients with asthma who were eligible for analysis. The characteristics of these patients are shown in Table 1. Of the 112 patients, 31 (28%) were also diagnosed with allergic rhinitis and/or chronic sinusitis by an otolaryngologist. There was a significant difference in age between patients with and without comorbid allergic rhinitis and/or chronic sinusitis. There was no difference in sex, smoking status, dose of ICS, and prevalence of treatment with long-acting $\beta_2$-agonists between the two groups.

### Air pollution on ADS and non-ADS days

In the 2011 season, ADS exposure occurred from May 1 to 3 in the four locations in the study, which are located within a radius of 200 km in a rural area of western Japan. A control non-ADS period was defined from 10–30 April 2011, just before the ADS period. The daily average levels of air pollutants in the ADS and non-ADS periods in each location are shown in Table 2. The average and daily maximum SPM differed significantly between ADS and non-ADS days in the four locations. In contrast, there were no significant differences in the levels of $SO_2$, $NO_2$, and $O_x$ between the ADS and non-ADS days in any of the locations.

Table 1. Characteristics of patients with or without comorbid allergic rhinitis (AR) and/or chronic sinusitis (CS).

| | All patients | Patients without comorbid AR and/or CS | Patients with comorbid AR and/or CS |
|---|---|---|---|
| Number | 112 | 81 | 31 |
| Age (year) | 61.4 ± 16.3 | 64.3 ± 14.7* | 55.5 ± 18.0* |
| Gender (male/female) | 43/69 | 31/50 | 12/19 |
| Allergic rhinitis/Chronic sinusitis/Both | 23/5/3 | 0/0/0 | 23/5/3 |
| Allergic conjunctivitis | 3 | 1 | 2 |
| Atopic dermatitis | 4 | 2 | 2 |
| Smoking status Never/Ex/Current | 101/8/3 | 72/6/3 | 29/2/0 |
| FVC (L) | 3.09 ± 0.91 | 3.00 ± 0.86 | 3.35 ± 0.96 |
| $FEV_1$ (L) | 2.45 ± 0.80 | 2.34 ± 0.75 | 2.66 ± 0.86 |
| $\%FEV_1$ (%) | 110.5 ± 18.5 | 106.4 ± 17.7 | 112.0 ± 23.5 |
| $FEV_1\%$ (%) | 79.2 ± 7.4 | 78.6 ± 6.7 | 79.1 ± 8.7 |
| Inhaled corticosteroid | 111 | 80 | 31 |
| Low dose | 27 | 15 | 12 |
| Medium dose | 73 | 60 | 13 |
| High dose | 11 | 5 | 6 |
| Long-acting $\beta_2$-agonists | 42 | 32 | 10 |
| Leukotriene receptor antagonist | 60 | 36 | 24 |
| Theophylline | 24 | 21 | 3 |
| Histamine antagonist | 26 | 1 | 25 |
| Intranasal corticosteroid | 12 | 0 | 12 |

Data is presented as the mean ± S.D.
FEV1; forced expiratory volume in 1 s, %FEV1; percentage of predicted FEV1, FVC; forced vital capacity.
*$p < 0.05$ by Mann–Whitney U for comparison of patients with and without comorbid allergic rhinitis and/or chronic sinusitis

Table 2. Daily average of air pollution levels in four locations during the ADS days (1–3 May 2011) and non-ADS days (10–30 April 2011).

| | Yonago City | | Matsue City | |
|---|---|---|---|---|
| | ADS days | Non-ADS days | ADS days | Non-ADS days |
| Av SPM, $\mu g/m^3$ | 80.3 ± 29.1* | 19.2 ± 1.5 | 64.0 ± 28.2* | 17.2 ± 1.5 |
| Av daily maximum SPM, $\mu g/m^3$ | 124.3 ± 2.1* | 38.3 ± 4.3 | 92.3 ± 13.8* | 29.4 ± 2.4 |
| Av $SO_2$, ppb | 1.0 ± 0.0 | 1.2 ± 0.1 | 0.6 ± 0.1 | 1.0 ± 0.1 |
| Av daily maximum $SO_2$, ppb | 2.0 ± 0.0 | 2.6 ± 0.4 | 1.1 ± 0.2 | 2.1 ± 0.3 |
| Av $NO_2$, ppb | 6.3 ± 3.1 | 6.1 ± 2.0 | 2.0 ± 1.1 | 2.5 ± 1.2 |
| Av daily maximum $NO_2$, ppb | 13.3 ± 8.5 | 15.3 ± 7.4 | 3.5 ± 1.9 | 5.9 ± 3.4 |
| Av Ox, ppb | 37.7 ± 5.0 | 38.3 ± 8.1 | 46.1 ± 12.4 | 53.1 ± 8.0 |
| Av daily maximum Ox, ppb | 52.3 ± 2.1 | 52.2 ± 13.3 | 61.5 ± 6.5 | 69.1 ± 11.6 |
| | Toyooka City | | Kanda Town | |
| Av SPM, $\mu g/m^3$ | 100.6 ± 6.5* | 22.2 ± 2.4 | 109.0 ± 35.8* | 28.8 ± 11.4 |
| Av daily maximum SPM, $\mu g/m^3$ | 162 ± 14.9* | 32.0 ± 11.8 | 160.0 ± 17.8* | 57.6 ± 33.2 |
| Av $SO_2$, ppb | 0.30 ± 0.06 | 0.71 ± 0.90 | 1.3 ± 0.6 | 2.1 ± 1.2 |
| Av daily maximum $SO_2$, ppb | 0.67 ± 0.33 | 1.3 ± 1.2 | 3.7 ± 2.1 | 5.9 ± 4.1 |
| Av $NO_2$, ppb | 3.86 ± 0.28 | 4.76 ± 0.48 | 10.0 ± 8.7 | 15.5 ± 10.7 |
| Av daily maximum $NO_2$, ppb | 13.0 ± 2.1 | 11.6 ± 3.7 | 26.0 ± 17.6 | 39.2 ± 22.4 |
| Av Ox, ppb | 45.2 ± 6.5 | 58.5 ± 2.1 | 37.0 ± 11.4 | 42.5 ± 14.4 |
| Av daily maximum Ox, ppb | 59.3 ± 6.4 | 67.0 ± 10.8 | 52.7 ± 13.7 | 63.8 ± 13.2 |

Data is presented as the mean ± S.D., Av; average, *$p < 0.05$, ADS days versus non-ADS days.

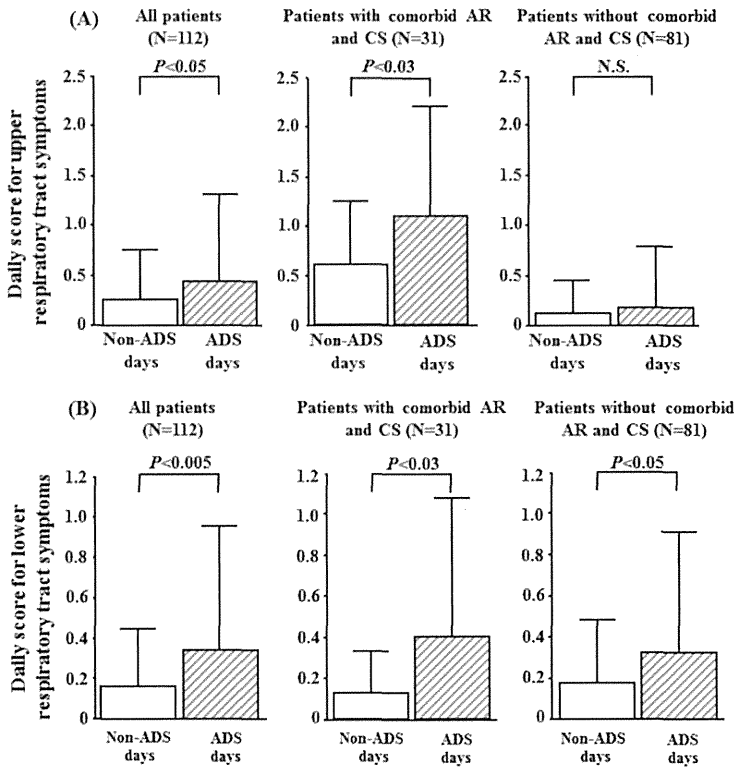


Figure 2. Scores for upper (A) and lower (B) respiratory tract symptoms in periods without (10–30 April 2011) and with (10–3 May 2011) an Asian Dust Storm (ADS) in all patients, and in patients with and without comorbid allergic rhinitis (AR) and/or chronic sinusitis (CS). Scores for upper respiratory tract symptoms such as stuffiness, sneezing, and lower respiratory tract symptoms such as cough, sputum, dyspnea, and wheezing were defined as 0 = absent, 1 = mild, and 2 = severe for each symptom.



### Respiratory symptom scores and PEF

In all patients, scores for upper respiratory tract symptoms were significantly higher on ADS days compared to non-ADS days ($0.45 \pm 0.08$ vs. $0.26 \pm 0.05$, $p < 0.05$; Figure 2A). Similarly, patients with comorbid allergic rhinitis and/or chronic sinusitis had significantly increased scores for upper respiratory tract symptoms on ADS days compared to non-ADS days (Figure 2A). In contrast, in patients without comorbid allergic rhinitis and/or chronic sinusitis, there was no significant difference in these scores on ADS and non-ADS days.

Scores for lower respiratory tract symptoms in all patients were also significantly higher on ADS days compared to non-ADS days ($0.35 \pm 0.61$ vs. $0.16 \pm 0.28$, $p < 0.005$; Figure 2B). There were significant differences in the scores for these symptoms between ADS and non-ADS days in patients with and without comorbid allergic rhinitis and chronic sinusitis (Figure 2B). Three patients made unscheduled hospital visits and took oral corticosteroids due to exacerbation of asthma without respiratory tract infection during the ADS period. In contrast, no patients needed oral steroids for exacerbation of asthma on non-ADS days, except for one event of exacerbation of asthma due to respiratory tract infection.

The mean morning PEF values were $384.9 \pm 89.6$ and $382.3 \pm 90.2$ l/min on ADS and non-ADS days (Figure 3A), with no significant difference between the periods. There were also no significant differences in mean morning PEF on ADS days compared to non-ADS days in patients with and without comorbid allergic rhinitis and/or chronic sinusitis (Figure 3A). The respective minimum morning PEF values were $92.4 \pm 5.9\%$ and $92.7 \pm 4.6\%$ in all patients, $93.4 \pm 3.2\%$ and $93.2 \pm 4.2\%$ in patients with comorbid allergic rhinitis and/or chronic sinusitis, and $92.3 \pm 5.1\%$ and $92.0 \pm 6.4\%$ in patients without comorbid allergic rhinitis and/or chronic sinusitis (Figure 3B), also without a significant difference.

### IL-8 transcriptional activity and IL-8 secretion in THP-G8 cells

The pH values of ADS airborne particles (400 μg/ml) and CJ-1 soil (400 μg/ml) were 7.9 and 8.4, respectively. THP-G8 cells were stimulated with CJ-1 soil after adjustment of the pH of the soil to 7.8 with 0.1 N sodium hydroxide. nSLO-LA was not increased before adjusting the pH (data not shown). Exposure of pH-adjusted CJ-1 soil ($n = 6$) also did not increase nSLO-LA in THP-G8 cells (Figure 4). In contrast, nSLO-LA was increased in exposure of cells to ADS airborne particles ($n = 6$) in a dose-dependent manner (Figure 5). Stimulation of THP-G8 cells with supernatant of ADS airborne particles caused nSLO-LA to increase to $1.3 \pm 0.1$ at 100 μg/ml and to $3.1 \pm 0.2$ at 400 μg/ml (Figure 5). However, the increase of nSLO-LA was smaller with the supernatant compared with the ADS airborne particles. The concentrations of IL-8 in supernatants of THP-G8 cells stimulated with vehicle, LPS ($n = 6$, 100 ng/ml), CJ-1 soil ($n = 6$, 400 μg/ml) and ADS airborne particles ($n = 6$,

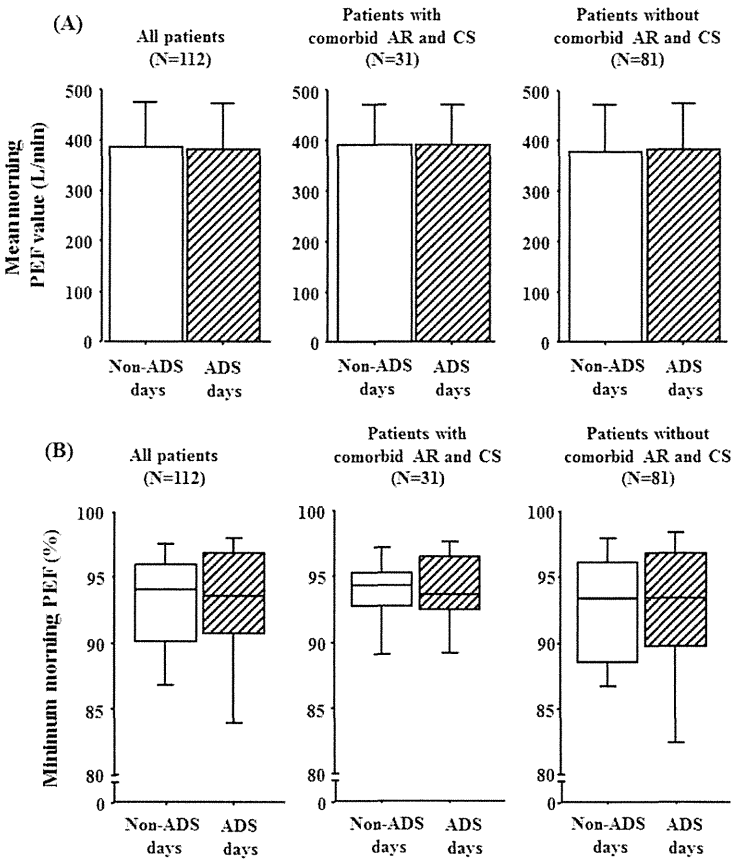


Figure 3. Morning peak expiratory flow (PEF) (A) and minimum morning PEF (% recent best) (B) in a period without (10–30 April 2011) and with (1–3 May 2011) an Asian Dust Storm (ADS) in all patients, and in patients with and without comorbid allergic rhinitis (AR) and/or chronic sinusitis (CS). The best PEF value was that recorded in three attempts within 30 min of waking up and before taking inhaled corticosteroids (ICS), $\beta_2$-agonists or oral drugs. The minimum morning PEF (% recent best) is defined as the lowest daily morning prebronchodilator PEF [26].



400 μg/ml) were 0.03 ± 0.002, 38.1 ± 1.7, 0.30 ± 0.004, and 29.7 ± 1.5 μg/ml, respectively (Figure 5).

### Endotoxin concentration in airborne particles collected on ADS days

The endotoxin concentrations were 0.192 EU/ml in ADS airborne particles at 400 μg/ml and 0.074 EU/ml in CJ-1 soil at 400 μg/ml. However, LPS at 100 ng/ml was out of the range of the assay, so we measured at 100 pg/ml of LPS. The endotoxin concentration was 0.885 EU/ml.

## Discussion

This study showed that exposure to an ADS aggravated upper and lower respiratory tract symptoms in adult patients with controlled asthma, with three patients having unscheduled hospital visits for exacerbation of asthma without respiratory tract infections on ADS days. IL-8 transcriptional activity in THP-G8 cells was stimulated by airborne particles collected on ADS days. These results suggest that asthma might be exacerbated by ADS exposure due to enhanced airway inflammation mediated by increased IL-8 in the airway.

Our previous telephone surveys showed that exposure to an ADS can aggravate lower respiratory tract symptoms and respiratory function in asthma patients [14,15]. In this study, we assessed both lower and upper respiratory tract symptoms on ADS and non-ADS days, and found that adult patients with asthma had worsened upper and lower respiratory tract symptom scores on ADS days. Three patients also had unscheduled hospital visits for asthma, although none were hospitalized, suggesting that ADS exposure has effects on symptoms in patients with controlled asthma. The worsening of upper respiratory tract symptoms in patient with asthma, and especially in those with comorbid allergic rhinitis and/or chronic sinusitis, on ADS days, is consistent with the findings in Min et al. [18]. In the present study, there were no significant differences in the mean morning PEF and the minimum morning PEF (% recent best) between ADS and non-ADS days, despite the worsening of symptoms. The lack of detection of a decrease in PEF on ADS days may have been due to the lower average level of SPM on these days in 2011 compared to our previous studies [14,15]. Alternatively, peak flow may not be a sensitive parameter for detecting subtle changes in asthma [29] and the patients in this study had well-controlled asthma.

IL-8 is a key chemokine in airway inflammation induced by air pollutants [23,24] and Honda et al. found that components adhered to ASD can increase release of IL-6 and IL-8 from airway epithelial cells [30]. An IL-8 promoter luciferase assay in THP-G8 cells, the so-called IL-8 Luc

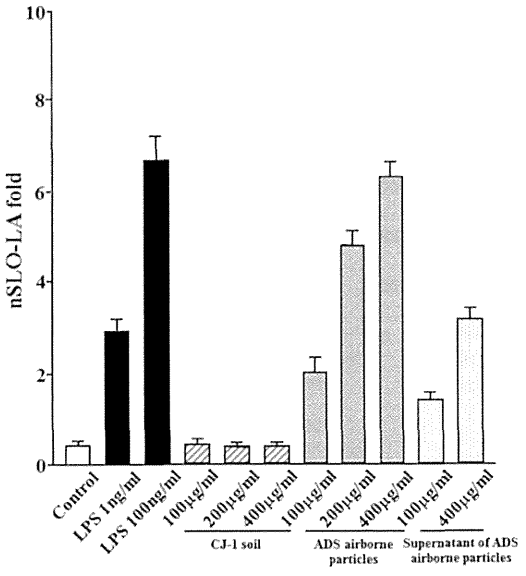


Figure 4. IL-8 transcriptional activity measured using an IL-8 luciferase assay in a stable THP-1-derived IL-8 reporter cell line. Cells were treated with solvent only (negative control), LPS (positive control), CJ-1 soil, airborne particles, and supernatant extracted from airborne particles collected during an ADS. IL-8 transcriptional activity was assessed from normalized SLO luciferase activity (nSLO-LA), which was calculated as SLO-LA divided by SLR-LA. The fold induction of nSLO-LA was calculated as the nSLO-LA of treated cells divided by that of untreated cells [27].

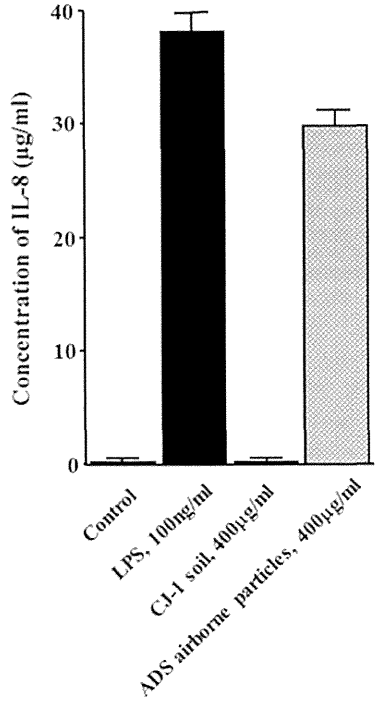


Figure 5. Concentration of IL-8 in supernatants of a stable THP-1-derived IL-8 reporter cell line stimulated with solvent only (negative control), LPS ($n = 6$, 100 ng/ml), CJ-1 ($n = 6$, 400 μg/ml), and dust particles collected during an ADS ($n = 6$, 400 μg/ml). The concentration of IL-8 was measured using an ELISA kit. Samples were run in triplicate. The range of the assay was from 31.2 to 2000 pg/ml.

assay, was used in the study because it has high sensitivity for evaluation of the IL-8 level using a small amount of material. Sand dust particles of ADS in Japan contain various chemicals, metals, microorganisms and ionic components [31,32]. To examine exacerbation of respiratory function by sand dust particles, we measured the difference in production of IL-8 between ADS airborne particles and the origin CJ-1 soil of the ADS. We found that ADS airborne particles promoted transcriptional activity and production of IL-8 in THP-G8 cells, with a significant increase in IL-8 transcriptional activity in THP-G8 cells treated with ADS airborne particles compared to those treated with original ADS soil. This difference may be caused by substances such as chemicals, metals and microorganisms carried by the ADS, in agreement with the findings of Honda et al. [30].

A large amount of precipitate was formed when an aqueous solution of airborne particles collected on ADS days was kept on ice for 1 h. The supernatant containing water-soluble ADS airborne particles also augmented IL-8 transcriptional activity in THP-G8 cells. This suggests that materials attached to or contained in the soil augmented this activity. Thus, ADS particles may exacerbate aggravation of asthma when chemicals, metals and microorganisms adhere to these particles.

Patients with asthma are more sensitive to the effects of environmental endotoxins (LPS) compared to healthy subjects, and inhaled LPS is associated with airway neutrophil inflammation in patients with asthma and in healthy subjects [33,34]. Our results showed that ADS airborne particles contained endotoxin, as also found previously [32], but the original soil did not do so, which suggests that endotoxin might have contributed to augmentation of the IL-8 transcriptional activity in THP-G8 cells.

There are several limitations in the study. First, we were not able to collect the same amount of airborne particles on non-ADS days because the level of SPM was very low on these days. Therefore, we were unable to compare stimulation of IL-8 transcription by airborne particles on ADS days with that by particles collected on non-ADS days. Second, we were not able to analyze the composition of airborne particles and the supernatant. Third, we did not assess the amount of pollen, which can affect upper respiratory tract symptoms in rhinitis patients from January to March, distinct from the period of ADS exposure. Future work is required to collect more airborne particles using two high-volume air samplers and a classification of the particles into four size ranges.

## Conclusion

We found that upper and lower respiratory tract symptoms in adult asthma patients worsened during a period of exposure to an ADS. Airborne particles collected on ADS days in western Japan, but not the origin soil of the ADS, increased IL-8 secretion in THP-G8 cells. This finding suggests that

exposure to an ADS may exacerbate asthma by enhancement of IL-8 transcriptional activity. Further studies are needed to better define the association between asthma and ADS.

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## Declaration of interest

None of the authors have a conflict of interest regarding the work in this study. The authors alone are responsible for the content and writing of this article. This study was supported by the Environmental Research and Technology Development Fund (C-1154) of the Japanese Ministry of the Environment.



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*Research Article*

# Decreased Pulmonary Function in School Children in Western Japan after Exposures to Asian Desert Dusts and Its Association with Interleukin-8

**Masanari Watanabe,[1] Hisashi Noma,[2] Jun Kurai,[1] Hiroyuki Sano,[3] Rumiko Saito,[4] Satoshi Abe,[5] Yutaka Kimura,[5] Setsuya Aiba,[5] Mitsuo Oshimura,[6] Akira Yamasaki,[1] and Eiji Shimizu[1]**

[1] *Department of Respiratory Medicine and Rheumatology, Tottori University Hospital, Yonago 683-8504, Japan*
[2] *Department of Data Science, Institute of Statistical Mathematics, Tokyo 190-8562, Japan*
[3] *Department of Respiratory Medicine and Allergology, Kinki University Faculty of Medicine, Osakasayama 589-8511, Japan*
[4] *Department of Integrative Genomics, Tohoku Medical Megabank Organization, Tohoku University, Sendai 980-8574, Japan*
[5] *Chromosome Engineering Research Center, Tottori University, Tottori 683-8504, Japan*
[6] *Department of Dermatology, Tohoku University Graduate School of Medicine, Sendai 980-8574, Japan*

Correspondence should be addressed to Masanari Watanabe; watanabm@grape.med.tottori-u.ac.jp

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The objective of the study was to investigate the influence of Asian dust storms (ADS) on pulmonary function of school children and the relationship of this effect with interleukin-8. Morning peak expiratory flow (PEF) was measured daily in 399 children from April to May 2012 and in 384 of these children from March to May 2013. The data were analyzed for an association between ADS events and PEF by linear mixed models. Interleukin-8 transcriptional activity was assessed in THP-G8 cells stimulated by airborne particles collected on ADS days. Seven ADS days were identified: April 23 and 24, 2012; March 8 to 10, 2013; and March 19 and 20, 2013. Changes in PEF after ADS exposure were −8.17 L/min (95% confidence interval, −11.40 to −4.93) in 2012 and −1.17 L/min (−4.07 to 1.74) in 2013, and there was a significant difference between 2012 and 2013. Interleukin-8 transcriptional activity was significantly higher in 2012 at 10.6 ± 2.9-fold compared to 3.7 ± 0.4 in March 8 to 10, 2013, and 2.3 ± 0.2 in March 19 and 20, 2013. The influence of ADS events on pulmonary function of children differs with each ADS event and may be related to interleukin-8 production.

## 1. Introduction

Asian dust storms (ADS) originating in the deserts of Mongolia, Northern China, and Kazakhstan often disperses dust over East Asia from spring until late autumn and is the second strongest source of dust emission worldwide [1]. An ADS is also a source of air pollutants because the dust contains chemicals, contaminating metals, microorganisms, and ionic components [2–4]. Therefore, ADS is a serious health problem associated with heavy pollution.

Numerous epidemiologic studies have shown that exposure to ADS increases rates of mortality, emergency treatment, and hospitalization for cardiovascular disease and pulmonary disease [5–8]. Other studies have shown that ADS increases the risk of hospitalization and exacerbates pulmonary function and respiratory symptoms in patients with asthma in Japan and South Korea [9, 10]. However, some studies from Taiwan have suggested that there is no significant association of ADS with asthma [11, 12]. Therefore, the influence of ADS on asthma may differ in different regions. This may be associated with differences in the materials attached to ADS airborne particles, which is influenced by the path the particles take [2–4]. We have also shown that effects on lower respiratory tract symptoms in adult patients with asthma differ for each ADS event [13]. Onishi et al. suggested that ADS events can be classified into three types

based on Lidar data: Type 1 events with high counts of air pollution aerosols, Type 2 events with high counts of mineral dust particles, in comparison to air pollution aerosols, and Type 3 events with very low counts of air pollution aerosols [4].

Neutrophils migrate to the lung during acute inflammation induced by exposure to air pollutants [14]. The concentration of interleukin-8 (IL-8) in bronchial lavage fluid and IL-8 mRNA expression in bronchial biopsy tissue from healthy subjects are also increased by air pollutants [15]. IL-8 is increased in the blood of asthma patients during exacerbation [16] and thus is thought to be a key cytokine in exacerbation of asthma. In this context, we found that airborne particles collected on ADS days in Western Japan induced production of IL-8 in THP-G8 cells, whereas this effect did not occur with the original soil of the ADS [17].

In 2012, a study was already conducted to investigate the influence of ADS and air pollutants on pulmonary function of school children in Western Japan. In the current study, to investigate the difference of the influence of ADS events on pulmonary function in children, we conducted an extended survey in 2013, which was to monitor daily peak expiratory flow (PEF) in the same children as the 2012 investigation. Each year, using an IL-8 luciferase assay, these related detrimental effects on pulmonary function and differences in IL-8 promoter activity induced by ADS airborne particles were studied.

## 2. Materials and Methods

*2.1. Subjects.* The aim of this longitudinal follow-up study was to examine effects of ADS events on pulmonary function in school children. Daily morning PEF of children was monitored from March to May 2012 and 2013 because ADS events are most frequent in these months. March 2012 was used as trial period to allow the children to familiarize themselves with the monitoring. There was no ADS event in March 2012. The study was performed in Matsue, the capital city of Shimane Prefecture, in Southwest Japan. The population of Matsue is about 200,000 and the area is 530.2 $km^2$. In March 2012, all 401 fourth grade students aged 8 to 9 years from 4 of 35 elementary schools in Matsue were enrolled in the study. The four elementary schools were within 10 km of each other and all subjects lived within a radius of 1 km of the schools.

The disposition of the children in the study is shown in Figure 1. A total of 401 children were recruited into the study in March 2012. Two were subsequently excluded due to failure to keep a daily record for PEF. Thus, records of daily PEF were analyzed for 399 children in 2012. In March 2013, we recruited the same 401 children, of whom one was excluded due to Moyamoya disease. Sixteen children were subsequently excluded due to failure to keep a daily record for PEF. Thus, records of daily PEF were analyzed for 384 children in 2013.

The subjects recorded their age, gender, height, weight, and presence of asthma, allergic rhinitis, allergic conjunctivitis, atopic dermatitis, and food allergy in March 2012 and March 2013. Subjects were defined as having asthma if they met any of the following criteria in the past 12 months: (1) diagnosis of asthma by a pediatrician, (2) wheezing, (3) use of asthma medication, and (4) visiting a hospital regularly

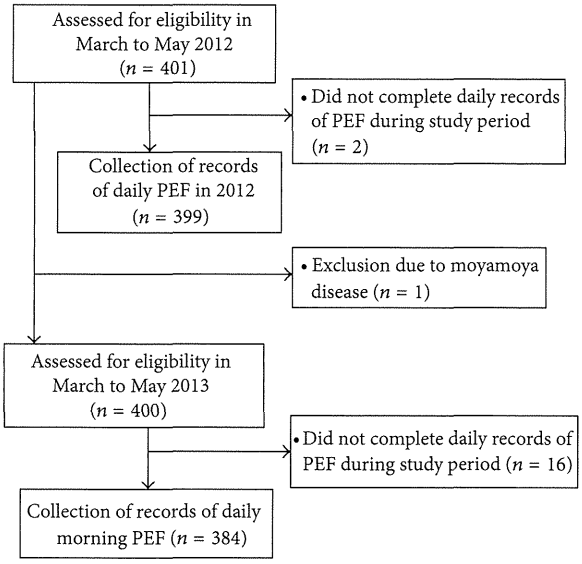


FIGURE 1: Flow chart showing the disposition of children in the study.

for asthma. Similarly, allergic rhinitis, allergic conjunctivitis, atopic dermatitis, and food allergy were judged to be present if the subjects met any of the following criteria in the past year: (1) diagnosis by a pediatrician, (2) use of medication for the disease, and (3) visiting a hospital regularly for the disease.

The study was approved by the institutional ethics committee (Ethics Committee of Tottori University, approval number 1764). We asked the Matsue City Board of Education for their help and received approval to submit the study to the schools. The study was also approved by the Parent Teacher Association (PTA) of each elementary school. Children and their parents were informed by teachers and we obtained formal consent for the study from the Matsue City Board of Education.

*2.2. Monitoring of PEF.* Before the study, the children and teachers were taught how to measure PEF. All children then measured their morning PEF daily using a peak flow meter (Mini-Wright, Harlow, England, American Thoracic Society Scale) from March to May 2012 and 2013, except for weekends and public holidays. Children recorded their best PEF value from three attempts after arriving at school between 8 a.m. and 9 a.m.

*2.3. Definition of ADS Days and Air Pollutant Monitoring.* The Japan Meteorological Agency has observatories throughout Japan and defines an ADS day based on a criterion of visibility <10 km due to dust arising from the deserts of East Asia, as determined by meteorological satellites monitoring each area. In this study, we used data from the Matsue observatory and we also referred to data from Light Detection and Ranging (Lidar) to define an ADS day. Lidar depolarization measurements performed simultaneously at two wavelengths can be used to identify nonspherical dust particles, which are mineral dust particles, and spherical aerosols such as organic

aerosols and inorganic sulfates and nitrates [19, 20]. Thus, Lidar can be used to measure levels of mineral dust particles as airborne sand dust particles and nonmineral dust particles as air pollution aerosols in real time. Lidar data are collected continuously in 23 locations in Japan, South Korea, China, Mongolia, and Thailand to detect a potential ADS. Other studies have defined ADS events as a daily (24-hour) average of mineral dust particles $>0.1\,km^{-1}$ [9] or $0.066\,km^{-1}$ (moderate ADS day) and $0.105\,km^{-1}$ (heavy ADS day) [21].

*2.4. Preparation of Airborne Particles Collected on ADS Days.* On ADS days, dust particles were collected at Tottori Prefectural Institute of Health and Environment, Yurihama, Tottori, which is located 70 km east of Matsue. This is the most suitable area close to Matsue to monitor particulate matter from East Asia because Yurihama is rural and has no source of air pollutants, except for motor vehicles. The observatory in Yurihama is also located away from populated areas. ADS airborne particles were collected in Tottori on April 23 and April 24, 2012; March 8 to 10, 2013; and March 19 and 20, 2013, using a high-volume air sampler (HV-1000R, Shibata, Tokyo, Japan) on the roof of a building. ADS airborne particles were filtered based on their aerodynamic diameters (Andersen Sampler, Shibata) into 5 sizes (<1.1, 1.1–2.0, 2.0–3.3, 3.3–7.0, and >7.0 $\mu$m) and each filter was dried in a desiccator before and after sampling to be weighed. ADS airborne particles of 3.3–7.0 $\mu$m were subsequently used in the study. Collected airborne dust was sterilized at 121°C for 30 min in an autoclave (Tomy SX-300, Tomy, Tokyo, Japan) to prevent growth of bacteria and fungi and dried at 80°C for 4 h with drying sterilizer (SG600, Yamato Scientific, Tokyo, Japan). The collected airborne dust was then weighed and stored in a freezer at −20°C. Soil from the China Loess Plateau (CJ-1), the original ADS soil from the Tengger Desert and Huining County located in Gansu Province, was obtained from the National Institute for Environmental Studies (Ibaraki, Japan) in 2002. This reference material is certified by the National Institute for Environmental Studies and the National Research Center for Environmental Analysis and Measurement (Beijing, China). To stimulate THP-G8 cells, airborne particles collected on ADS days were diluted to 1 mg/mL with distilled deionized water.

*2.5. IL-8 Promoter-Luciferase Gene Reporter Assay and Measurements of IL-8 and Endotoxin.* THP-G8 cells are a THP-1-derived reporter cell line that express stable luciferase orange (SLO) and stable luciferase red (SLR) genes under control of the IL-8 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) promoters, respectively [22]. The THP-G8 cell line was kindly provided by the Department of Dermatology, Tohoku University Graduate School of Medicine, Sendai, Japan, and was cultured as described previously [22]. We first stimulated THP-G8 cells ($5 \times 10^4$ cells/100 $\mu$L/well) in 96-well black plates (Greiner Bio-One GmbH, Frickenhausen, Germany) with lipopolysaccharide (LPS) (Wako Pure Chemicals, Osaka, Japan) and examined IL-8 and GAPDH reporter activity for various time periods and concentrations. Luciferase activity was determined using a microplate luminometer with a Phelios multicolor detection system (Atto, Tokyo, Japan) using Tripluc luciferase assay reagent (Toyobo, Osaka, Japan). IL-8 transcriptional activity was assessed from normalized SLO luciferase activity (nSLO-LA), which was calculated as SLO-LA divided by SLR-LA, and the fold induction of nSLO-LA was calculated as the nSLO-LA level of treated cells divided by that of untreated cells [22]. Induction of IL-8 transcriptional activity was measured after THP-G8 cells were stimulated for 5 h with solvent only (negative control), 100 ng/mL lipopolysaccharide (LPS), and ADS airborne particles collected in 2012 and 2013.

IL-8 concentrations in culture supernatants were determined using an enzyme-linked immunosorbent assay (ELISA) kit for IL-8 (R&D Systems, Minneapolis, MN, USA). Samples were run in triplicate and read using an automated ELISA reader (Model 680, Bio-Rad, Philadelphia, PA, USA). The range of the assay was 31.2 to 2000 pg/mL. Endotoxin concentrations in ADS airborne particles were measured using a chromogenic LAL endotoxin assay kit (GenScript, Piscataway, NJ, USA). The range of the assay was 0.01 to 1 EU/mL. The pH of ADS airborne particles was measured with a pH meter (MP220, Mettler Toledo, Schwerzenbach, Switzerland).

*2.6. Measurement of Metal Elements in CJ-1 Soil and ADS Airborne Particles.* Metal elements in CJ-1 soil and ADS airborne particles collected on April 23 and 24, 2012; March 8 to 10, 2013; and March 19 and 20, 2013 were measured by Oki Engineering (Tokyo, Japan). The concentrations of aluminum (Al), arsenic (As), barium (Ba), calcium (Ca), cadmium (Cd), cobalt (Co), chromium (Cr), copper (Cu), iron (Fe), mercury (Hg), potassium (K), lanthanum (La), magnesium (Mg), manganese (Mn), sodium (Na), nickel (Ni), phosphorus (P), lead (Pb), strontium (Sr), titanium (Ti), and zinc (Zn) were measured by inductively coupled plasma atomic emission spectrometry. Silicon (Si) was measured using electrothermal atomic absorption spectrometry.

*2.7. Statistical Analysis.* To evaluate the effects of exposure to an ADS on the daily PEF of children, linear mixed models that accounted for correlations among repeated measurements within a subject were used to estimate the effects of exposure to an ADS on the daily PEF of children in April to May 2012 and March to May 2013 [23, 24]. Additionally, to adjust for potential confounding factors, we used linear mixed models with the following general form:

$$Y_{ij} = \beta_0 + \beta_1 x_{1,j} + \sum_{k=1}^{p} \beta_k x_{k,ij} + b_{0,i} + \varepsilon_{ij}. \quad (1)$$

$Y_{ij}$ corresponds to the daily PEF for the $i$th child at the $j$th day ($i = 1, 2, \ldots, N$; $j = 1, 2, \ldots, T$). $x_{1,j}$ is an exposure variable of $j$th day (measurement of air pollution), and $x_{k,ij}$ ($k = 2, 3, \ldots, p$) are potential confounding factors involving individual characteristics (age, gender, height, weight, and presence of asthma, allergic rhinitis, allergic conjunctivitis, atopic dermatitis, and food allergies) and meteorological variables such as daily temperature, humidity, and atmospheric pressure. $\beta_0, \beta_1, \ldots, \beta_p$ are corresponding fixed effects coefficients, and $b_{0,i}$ is the random effect of intercept

Table 1: Characteristics of children.

| | 2012 | 2013 |
|---|---|---|
| Number | 399 | 384 |
| Gender (male/female) | 205/194 | 194/190 |
| Height (cm) | 132.3 ± 5.9 | 137.7 ± 7.0 |
| Male | 132.2 ± 5.5 | 136.9 ± 6.3 |
| Female | 132.4 ± 6.4 | 138.5 ± 7.7 |
| Weight (kg) | 29.5 ± 5.8 | 32.4 ± 6.6 |
| Male | 29.6 ± 6.2 | 32.3 ± 6.8 |
| Female | 29.3 ± 5.4 | 32.6 ± 6.4 |
| Allergic disease | | |
| Asthma | 38 | 45 |
| Allergic rhinitis | 78 | 74 |
| Allergic conjunctivitis | 8 | 15 |
| Atopic dermatitis | 44 | 36 |
| Food allergy | 19 | 20 |

Data are shown as the mean ± S.D.

for $i$th child and are assumed to be $b_{0,i} \sim N(0, \sigma_b^2)$. $\varepsilon_{ij}$ is the error term, $\varepsilon_{ij} \sim N(0, \sigma^2)$. In addition, effects on PEF were measured from the day of ADS exposure until 3 days after exposure because a dust effect on PEF can persist for up to 3 days [10]. Differences in PEF between the 2012 and 2013 results were also evaluated. The two-pollutant model was applied to different combinations of pollutants to assess the stability of the effects of ADS on PEF after adjustment for individual characteristics (age, gender, height, weight, and presence of asthma, allergic rhinitis, allergic conjunctivitis, atopic dermatitis, and food allergies) and meteorological variables (temperature, humidity, and atmospheric pressure). R version 3.0.3 (R Foundation for Statistical Computing, Vienna, Austria) was used for statistical analysis of PEF values and ADS exposure. Differences of nSLO-LA of THP-G8 cells were analyzed by ANOVA using SPSS Statistics (Japanese version 21.0 for Windows, IBM Japan, Tokyo, Japan). All quoted $P$ values are two-sided and the significance level was set to 0.05.

## 3. Results

*3.1. Profile of the Children.* The characteristics of the children in the 2012 and 2013 studies are shown in Table 1.

*3.2. Air Pollution Levels and Weather Information on ADS Days and Non-ADS Days.* In 2012, April 23 and 24 were identified as ADS days. In 2013, March 8 to 10 and 19 and 20 were similarly identified as ADS days. Non-ADS days were defined as all other days from April 1 to May 31, 2012, and from March 1 to May 31, 2013. Daily levels of mineral dust particles (airborne sand dust particles) and suspended particulate matter (SPM) are shown in each period in Figure 2. The levels of air pollutants and weather during the study periods are shown in Table 2.

*3.3. PEF.* Changes in PEF after exposure to ADS are shown in Figure 3. In order to show the post-ADS-exposure effects,

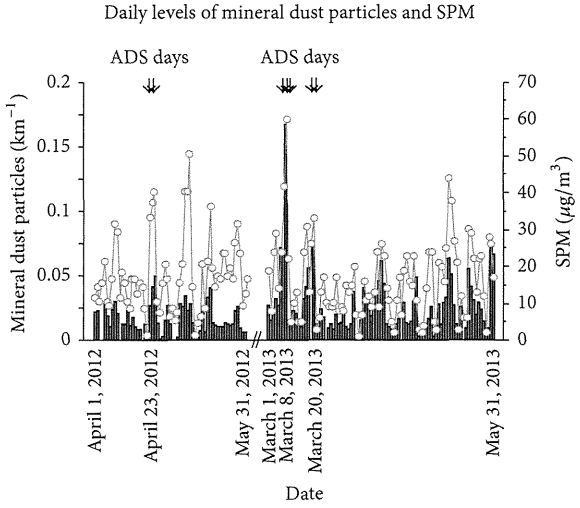


Figure 2: Daily levels of mineral dust particles (airborne sand dust particles) (bar graph) and SPM (line graph). Arrows indicate ADS days.

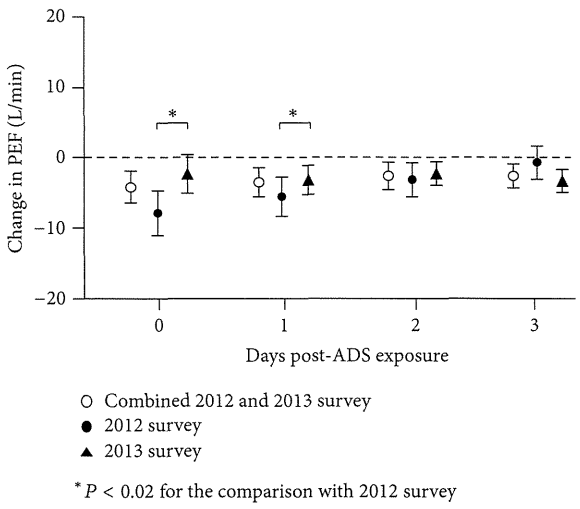


Figure 3: PEF changes caused by an ADS event from 0 (ADS day) to 3 days after ADS exposure in combined 2012 and 2013 (open circles), 2012 (black circles), and 2013 (triangles), with 95% confidence intervals (error bars). Data are controlled for age, gender, height, weight, and presence of asthma, allergic rhinitis, allergic conjunctivitis, atopic dermatitis, and food allergies; meteorological variables such as daily temperature, humidity, and atmospheric pressure; and the linear time trend. There are significant differences in the decrement of PEF on days 0 and 1 between 2012 and 2013 (*$P$ < 0.02).

these changes are shown from 0 (ADS day) to 3 days after ADS exposure. In combining 2012 and 2013, the changes in PEF after exposure to ADS exposure were −4.16 L/min (95% CI, −6.33 to −1.99) on day 0, −2.97 L/min (−5.03 to −0.91) on day 1, −2.23 L/min (−4.12 to −0.35) on day 2, and −2.57 L/min (−4.29 to −0.86) on day 3 after ADS. There were significant decreases in PEF from day 0 to day 3 after ADS exposure. In 2012, the changes in PEF were −7.82 L/min (−10.93 to −4.71)