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Letter

Cytometric analysis on cytotoxicity of 4,4′-methylenediphenyl diisocyanate, a chemical allergen, in rat thymocytes

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ABSTRACT — 4,4'-Methylenediphenyl diisocyanate (MDI) is a cross-linking agent. Chemical reactivity of MDI with endogenous substances such as albumin and glutathione (GSH) is assumed to be responsible for MDI toxicity. We examined the cytotoxic effect of MDI on rat thymocytes, under the condition that endogenous biological substances except for cells were nominally absent, in order to study chemico-biological interactions between MDI and cells. The treatment of 10-50 μM MDI for 3 hr significantly increased the side scatter signal intensity of cytograms, without affecting forward scatter intensity. The increase in side scatter signal intensity by MDI was associated with an increase in cell lethality. The treatment of cells with 50 μM MDI for 3 hr increased cell lethality without increasing the population of pre-apoptotic annexin V-positive living cells. In contrast, H_2O_2 at 100 μM significantly increased the population of annexin V positive living cells prior to cell death. MDI at 30-50 μM did not affect the increase in cell lethality induced by H_2O_2 or A23187. Simultaneous application of 50 μM GSH did not affect the cytotoxicity of 50 μM MDI. It was therefore concluded that the process of cell death induced by MDI could not be attributed to oxidative stress and intracellular Ca^{2+} overload, and that MDI possesses cytotoxic actions that are not significantly related to its chemical reactivity with GSH.

Key words: 4,4′-Methylenediphenyl diisocyanate, Cytotoxicity, Lymphocytes, Flow-cytometer

INTRODUCTION

4,4'-Methylenediphenyl diisocyanate (MDI) is primarily used as a cross-linking agent to produce rigid polyurethane and industrial adhesives. This low weight chemical is an allergen that may lead to asthma in workers who are chronically exposed (Mapp *et al*., 1988; Nakashima *et al*., 2002). Various studies have indicated that the chemical reactivity of MDI with endogenous substances such as albumin and glutathione (GSH) is responsible for its allergenic properties. Isocyanate-specific adducts of MDI with hemoglobin and albumin are involved in the etiology of the sensitization reaction (Sabbioni *et al*., 2010; Gries and Leng, 2013). MDI reactivity with GSH releases by-products that stimulate pathogenic inflammatory processes such as macrophage activation and eosinophilic airway inflammation (Wisnewski *et al*., 2013). Although there are many reports concerning occupational asthma caused by MDI, information on the cellular actions of MDI is very limited. *In vitro* studies have revealed that MDI caused cell death and DNA double-strand breaks in human lung epithelial cell line A549 (Vock *et al*., 1998), and induced chromosomal aberrations in cultured human lymphocytes (Mäki-Paakkanen and Norppa, 1987) as well as Chinese hamster fibroblasts (Ishidate, 1988). In addition, MDI elicited the release of the chemokine interleukin-8 in the human promyelocytic cell line THP-1 (Mitjans *et al*., 2008). These studies indicate that MDI directly reacts with cell membranes and organella, leading to cytotoxic actions.

In order to examine the cytotoxic effects of MDI on rat thymic lymphocytes in the absence of endogenous bio-

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logical substances, thymic cells were initially purified. We studied the effect of MDI on cells suffering from oxidative stress or Ca^{2+} overload to ascertain whether MDI increases cell vulnerability to chemical stress. Furthermore, we examined the cytotoxicity of MDI in the presence of GSH. By these means, we aimed to elucidate the chemico-biological interactions between MDI and cells, which may subsequently clarify its links to occupational allergy and asthma.

MATERIALS AND METHODS

Chemicals

MDI (purity 99.7 %) was supplied by Tokyo Chemical Industry (Tokyo, Japan). Propidium iodide (PI), annexin V-FITC, and 5-chloromethylfluorescein diacetate (5CMF) were purchased from Invitrogen (Eugene, OR, USA). Fluo-3-AM was obtained from Dojindo (Kumamoto, Japan). A23187 was obtained from Sigma Chemicals (St. Louis, MO, USA). Other chemical compounds were supplied by Wako Pure Chemicals (Osaka, Japan) unless stated otherwise.

Animals and cell preparation

Experimental approval (No. 14124) was obtained from the Tokushima University Committee for Animal Experiments (Tokushima, Japan). Cell suspensions were prepared as follows: (1) Wistar male rats were anesthetized with ether; (2) slices of thymus glands were triturated in Tyrode's solution (2-4°C, 150 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM glucose, pH 7.3-7.4 adjusted by 5 mM HEPES and NaOH) to dissociate thymocytes; (3) the Tyrode's solution was passed through a stainless steel mesh to prepare the cell suspension; (4) the cells were incubated at 36-37°C for 1 hr before the experiment. This preparation is suitable for flow-cytometric cytotoxicity test because single dissociated cells with intact membranes are obtained because no enzymatic treatment is required to isolate single cells.

Protocols for *in vitro* experiments

Various concentrations of MDI (10-100 mM in 2 µL dimethyl sulfoxide) were added to the cell suspensions (2 mL per test tube), and the cells were treated with MDI at 36-37°C for 2-3 hr. A sample (100 µL) was analyzed by a flow cytometer (CytoACE-150; JASCO, Tokyo, Japan) to assess the MDI-induced actions on membrane and cellular parameters using JASCO software (Version 3.06). Data acquisition from 2.5×10^3 cells required 10-15 sec.

Fluorescence measurements of membrane and cellular parameters

To assess cell lethality, PI was added to the cell suspensions to achieve a final concentration of 5 µM. PI fluorescence provides information on cell lethality. The fluorescence was measured 2 min after the application of PI. Exposure of phosphatidylserine (PS) on outer membrane surfaces was detected using annexin V-FITC, as previously described (Koopman *et al*., 1994). The cells were incubated with annexin V-FITC (10 µL/mL) for 30 min before measurement. To estimate change in intracellular Ca^{2+} level ($[Ca^{2+}]_i$)with Fluo-3 fluorescence (Kao *et al*., 1989), the cells were incubated with 1 µM Fluo-3-AM for 1 hr before any fluorescence measurement. Cellular content of GSH ($[GSH]_i$) was performed with 5CMF fluorescence (Chikahisa *et al*., 1996). 5CMF-DA at 500 nM was applied to the cell suspension 30 min before the measurement. The excitation wavelength for fluorescent probes was 488 nm, while emissions were detected at 530 ± 20 nm for FITC, 5CMF, and Fluo-3 fluorescence and at 600 ± 20 nm for PI fluorescence, respectively. There was no background fluorescent signal from the reagents used under our experimental conditions.

Statistical analysis

Statistical analyses were performed through an analysis of variance, with post hoc Tukey's multivariate analysis. *P*-values (< 0.05) were considered statistically significant. Each experiment was repeated three times unless noted otherwise.

RESULTS

MDI-induced change in cytograms (forward scatter *versus* side scatter)

The cells were treated with MDI for 3 hr. Fig. 1 shows the effects of 50 µM MDI. There were cells with an increased intensity of side scatter and those with decreased intensity of forward scatter (Fig. 1A). The shrunken cells (those displaying a reduced intensity of forward scatter) exhibited PI fluorescence, indicating that they were dead (Fig. 1B). Increases in the side scatter intensity of both cells with and without PI fluorescence (viable and dead cells) were observed (Fig. 1B).

MDI-induced increase in population of cells stained with PI

Thymocytes were incubated with varying concentrations of MDI (10-100 µM) for 3 hr. MDI at concentrations of 30-100 µM significantly increased the population of cells exhibiting PI fluorescence, indicating a dose-de-

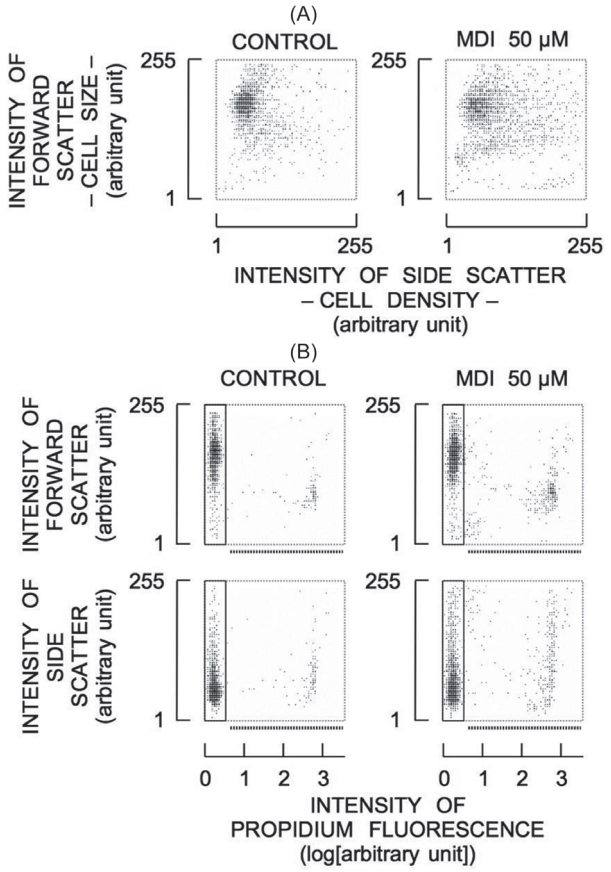

Fig. 1. MDI-induced changes in cytograms. (A) Change in cytogram forward scatter *versus* side scatter by the treatment of cells with 50 μM MDI for 3 hr. (B) MDI-induced changes in cytograms (forward or side scatter *versus* propidium fluorescence, upper and lower panels, respectively). The dotted line under each cytogram in (B) indicates cells exhibiting propidium fluorescence (i.e., dead cells). The area enclosed within a square indicates the population of living cells. Cytograms were constructed with 2500 cells.

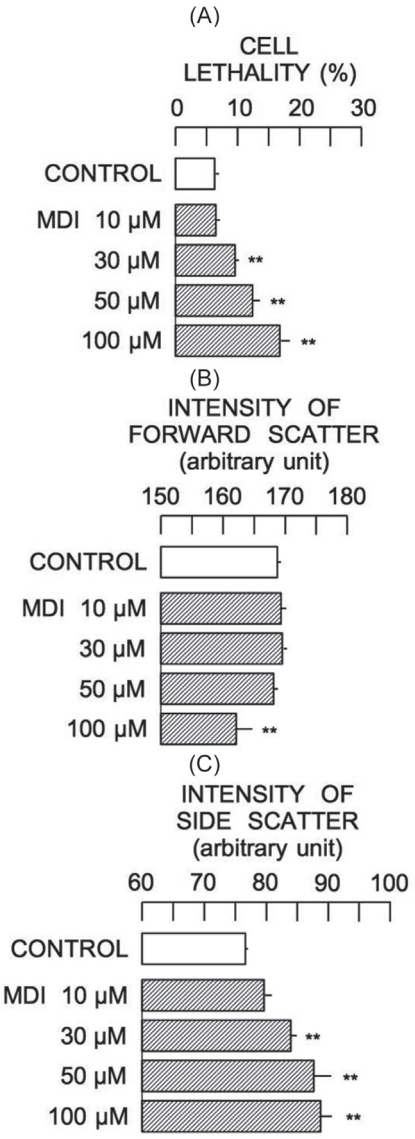

Fig. 2. Concentration-dependent changes in cell lethality (A), forward scatter intensity (B), and side scatter intensity (C) by the treatment of cells with 10-100 μM MDI for 3 hr. Columns and bars represent the means and standard deviation, respectively, of four samples. Asterisks (**) indicate significant difference ($P < 0.01$) between the MDI-treated groups and non-treated control (CONTROL).

pendent MDI-induced increase in cell lethality (Fig. 2A).

MDI-induced changes in the forward and side scatter signal intensity of living cells

Mean forward scatter signal intensity was attenuated by the incubation with 100 μM MDI for 3 hr (Fig. 2B), indicating a slight, but statistically significant, decrease in cell size by 100 μM MDI. MDI at concentrations of 50 μM or less did not produce this effect. Mean side scatter signal intensity was significantly augmented by the incubation with 30-100 μM MDI in a dose-dependent manner (Fig. 2C). The lower dose of 10 μM MDI however, did not have a significant effect. This result shows the significant increase in cell density (cell granularity) by 30-100 μM DMI.

MDI-induced change in cell groups classified with PI and annexin V-FITC

Further analysis of MDI-induced cytotoxicity was

performed with annexin V-FITC and PI. As shown in Fig. 3A, there were four groups (*N*, *A*, *P*, and *AP*) in the cytogram (PI fluorescence *versus* FITC fluorescence). Treatment of cells with 100 μM H_2O_2 as a positive control for chemical-induced oxidative stress greatly decreased intact living cells (group *N*) and increased annexin V-positive living cells (group *A*). Annexin V-positive dead cells (group *AP*) were also increased by H_2O_2. However, only a few cells were categorized to group *P* (annexin V-negative dead cells). Cells treated with 50 μM MDI for 3 hr displayed different features; the group of intact living cells (MDI group *N*) largely remained 3 hr after MDI application, however there was a significant reduction in comparison with control group *N* (Fig. 3A and B). There was no difference in the population of annexin V-positive living cells (group A) between MDI-treated and non-treated samples (Fig. 3B). The population of dead cells (group AP+P) was increased by MDI (Fig. 3B). Interestingly, there was a small population of annexin V-negative dead cells in MDI group *P* (Fig. 3A). It was not the cases of control and H_2O_2-treated groups.

Effect of MDI on cells treated with H_2O_2 or A23187

Oxidative stress and intracellular Ca^{2+} overload can induce cell death (Orrenius *et al*., 2015). To see if MDI is able to modulate oxidative stress or Ca^{2+} overload-induced cytotoxicity, the effects of MDI on cells treated with H_2O_2 and A23187 were examined. The incubation of cells with 100 μM H_2O_2 or 100 nM A23187 for 3 hr increased cell lethality by 20-30% (Fig. 4A), in comparison with a 10-20% increase by 30 μM and 50 μM MDI (Fig. 2A). The combination of MDI and H_2O_2 or MDI and A23187 elicited only an additive increase in cell lethality, as shown in Fig. 4A. Therefore, the process of cell death induced by MDI may be different from that induced by oxidative stress and Ca^{2+} overload.

Cytotoxicity of MDI in the presence of GSH

It has been previously demonstrated through *in vivo* experiments that MDI reacts with GSH (Wisnewski *et al*., 2013). Therefore, GSH may attenuate the cytotoxicity of MDI by reducing the reactivity of MDI at cell level. The cytotoxicity of 50 μM MDI to thymocytes was thus tested in the presence of 50 μM GSH. Simultaneous application of GSH did not change the cytotoxicity of 50 μM MDI (Fig. 4B).

Effects of MDI on 5CMF and Fluo-3 fluorescence

The treatment of living cells with 10-50 μM MDI for 1 hr did not significantly affect 5CMF and Fluo-3 flu-

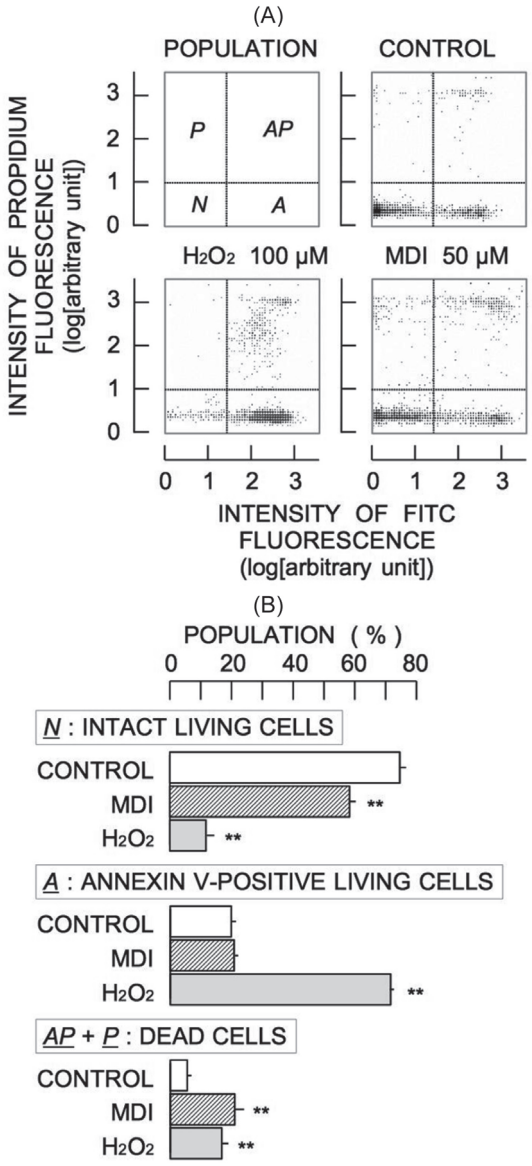

Fig. 3. Changes in propidium iodide and annexin V-FITC signal scatter of cells treated with MDI and H_2O_2. Isolated thymocytes were incubated with 100 μM H_2O_2 or 50 μM MDI for 3 hr. The probes were added to cell suspensions at 30 min before measurement. (A) Changes in cytogram scatter (PI fluorescence *versus* FITC fluorescence) in H2O2-treated and MDI-treated cells. Each cytogram was constructed with 2500 cells. Areas marked "N", "A", "P", and "AP" refer to populations of intact living cells, annexin V-positive living cells, dead cells, and annexin V-positive dead cells, respectively. (B) Percentage population of cells classified with PI and annexin V-FITC. Columns and bars represent the means and standard deviation, respectively, of four samples. Asterisks (**) indicate a significant difference ($P < 0.01$) between H2O2-treated / MDI-treated groups and the non-treated control (CONTROL).

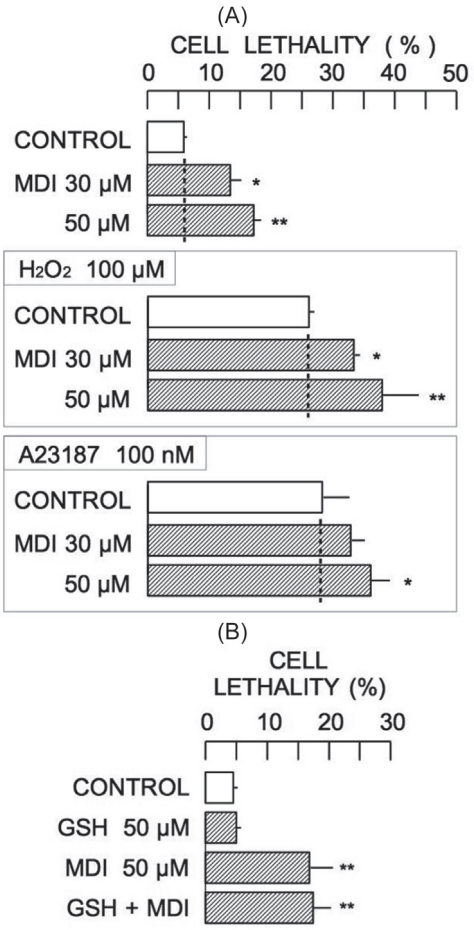

Fig. 4. Effects of MDI on cells simultaneously treated with H_2O_2, A23187, or GSH. (A) Effect in the presence of H_2O_2 or A23187. Upper panel: percentage lethality of cells treated with MDI alone. Middle and lower panels: percentage lethality of cells co-treated with MDI and 100 μM H_2O_2 (middle) or 100 nM A23187 (lower). Columns and bars represent means and standard deviation, respectively, of four samples. Asterisks (* and **) indicate a significant difference ($P < 0.05$ and 0.01, respectively) between the control group (CONTROL) and drug-treated groups. (B) Cytotoxicity of MDI in the presence of GSH. Cytotoxicity is displayed as a percentage of dead cells after treatment with MDI, GSH or a combination of both, in comparison to non-treated cells. Columns and bars represent means and standard deviation, respectively, of four samples. Asterisks (**) indicate a significant difference ($P < 0.01$) between the control group (CONTROL) and drug-treated groups.

orescence. Results indicate that MDI did not change $[GSH]_i$ and $[Ca^{2+}]_i$.

DISCUSSION

MDI at concentrations of 10-100 μM increased the intensity of side scatter of living thymocytes, which is indicative of an increase in cell granularity. Therefore, the treatment of cells with MDI for 3 hr affects intracellular circumstance of living cells. The MDI-induced change in cell granularity was not associated with any changes in cell volume, as determined by forward scatter signal intensities, a parameter for cell size. The correlation between side scatter signal intensity and cytotoxic action has not been elucidated, although some cytotoxic substances have been reported to significantly augment side scatter (Kamae *et al*., 2017; Imai *et al*., 2017; Mitani *et al*., 2017).

The pathway of cell death induced by MDI appeared to be different from that induced by H_2O_2 and A23187 (i.e. oxidative stress and intracellular Ca^{2+} overload), according to several observations. First, there were several differences in the scatters of cytograms classified by PI and annexin V-FITC between cells treated with MDI and H_2O_2. The cytogram (PI fluorescence *versus* FITC fluorescence) of cells treated with H_2O_2 was similar to that with A23187 (Sakanashi *et al*., 2008). Secondly, MDI did not potentiate the cytotoxicity of H_2O_2 and A23187. Both H_2O_2 and A23187 increased the population of annexin V-positive living cells, while MDI did not. We further ascertained that apoptosis was not the primary mechanism by which MDI induced cell death as there was no difference in the population of annexin V-positive living cells, an early stage during apoptosis, between control and MDI-treated cells. Thirdly, MDI did not increase $[Ca^{2+}]_i$. Finally, MDI did not decrease $[GHS]_i$. If MDI induces oxidative stress, the treatment of cells with MDI would reduce $[GSH]_i$.

Isocyanates are known to react with endogenous substances (Sabbioni *et al*., 2010; Gries and Leng, 2013), and MDI in particular has demonstrated reactivity with GSH (Wisnewski *et al*., 2013). The chemical reactivity of MDI is considered responsible for its toxicity to cells (Wisnewski *et al*., 2013). However, the co-treatment of cells with MDI and equimolar GSH did not change the cytotoxicity of MDI. Therefore, MDI may possess cytotoxic actions that are not significantly related to chemical reactivity with GSH. The physiochemical property of MDI is probably a key point for this study. The water solubility of MDI is very low. Although isocyanates including MDI are considered to be unstable compounds, it is considered to be relatively stable if nucleophilic compounds are absent. GSH, DMSO, glucose, and H_2O were conceiva-

ble nucleophiles in the solution except for the cells. Since MDI was initially dissolved in DMSO, the nucleophilicity of MDI in DMSO is considered to be not strong. Thus, MDI is relatively stable under present experimental conditions. The nucleophilicity is also related to the pH of aqueous solution. If it is basic, the nucleophilicity should be dramatically increased. The change is little around pH 7. GSH, DMSO, and glucose are soluble in water. DMSO is arbitrarily mixed with H_2O. Thus, GSH and glucose dissolve in the H_2O-DMSO solution. However, since the solubility of MDI is quite low, MDI attaches to lipophilic membranes of thymocytes. The reaction of MDI with GSH is considerably slowed. Furthermore, GSH is considered to form a complex with metal ions in the solution. If GSH forms a complex with metal divalent cations, the nucleophilicity would be considerably reduced.

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Conflict of interest---- The authors declare that there is no conflict of interest.

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