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Original Article

Stimulation of Hydrogen Peroxide Production by Drinking Water Contaminants in HL-60 Cells Sensitized by Retinoic Acid

Hideaki YOSHIDA*1, Shoichi INOUE*1, Kohki YOSHIDA*2, Osamu NAKAJIMA*3 and Shigetaka MIZUNO*4

*1 *Department of Environmental Medicine and Informatics, Graduate School of Environmental Earth Science, Hokkaido University, Sapporo*
*2 *Department of Hematology, National Sapporo Hospital, Sapporo*
*3 *Division of Laboratory Medicine, Hokkaido University Hospital, Hokkaido University, Sapporo*
*4 *Central Clinical Laboratory, Hokkaido University Dental Hospital, Hokkaido University, Sapporo*

Abstract

Chemical carcinogens, such as chloroform and trichloroethylene, are present in drinking water in Japan. As these contaminants are believed to have a role in carcinogenesis, we examined if chloroform and trichloroethylene, as well as methylene chloride, xylene, benzene, and ethanol, have the ability to generate hydrogen peroxide (H_2O_2) in human polymorphonuclear leukocytes (PMN) and human leukemia (HL-60) cells. Methylene chloride, benzene, xylene, trichloroethylene, and ethanol did not increase cellular H_2O_2 production as measured by flow cytometry nor as observed by confocal laser microscopy. In PMN and RA-untreated HL-60 cells chloroform did not significantly affect H_2O_2 levels. However, in HL-60 cells sensitized by pretreatment of 10 nM retinoic acid (RA) for 12 h, chloroform induced a significant increase in H_2O_2, but the increase induced by trichloroethylene was not significant. The observed increase in fluorescence was confirmed using a confocal laser microscope. These results indicate that chloroform and trichloroethylene may stimulate H_2O_2 production in HL-60 cells sensitized by pretreatment of RA. Our method may be useful to test if weak stimulants can stimulate intracellular H_2O_2 production.

Key words: Hydrogen peroxide, Flow cytometry, Retinoic acid, Differentiated leukemia cell, Carcinogenicity

Introduction

A number of organic compounds contaminate sources of water supply. Because these chemical compounds are not necessarily eliminated by usual techniques, such as chlorination, coagulation and filtration[1], drinking water contaminated with these chemical pollutants is being supplied in Japan. Trihalomethanes have been found in chlorinated drinking water in Cairo[2]. Chloroform, one of the trihalomethanes, and trichloroethylene were shown to be carcinogenic in animals and humans[3,4].

Evidence exists to suggest that tumor promotion may be associated with inflammation[5]. In the inflammatory process initiated by bacteria or chemicals, inflammatory cells, including neutrophils and macrophages, accumulate at the inflammatory lesion. These cells produce oxygen radicals. Moreover, in inflammatory cells previously activated (priming) by a stimulant, such as cytokines, the oxygen radical generation is easily accelerated by other stimulants[6,7]. These oxygen radicals consequently result in damage to genetic material and may lead to malignant transformations of cells damaged genetically. Hence, oxidant damage of genetic material may contribute to carcinogenesis[5].

The major species of reactive oxygen are the superoxide ($O_2^{\cdot}$) and its conjugate acid, the perhydrooxy radical ($HO_2^{\cdot}$), singlet oxygen ($^1O_2$), the hydroxyl radical ($OH^{\cdot}$), and hydrogen peroxide (H_2O_2)[8]. The covalent binding of many carcinogens to DNA requires activation to electrophilic, ultimate metabolites[9,10]. These carcinogens cause DNA damage directly or indirectly[11]. Carcinogenic compounds acting indirectly on DNA produce DNA-damaging agents through reactions with cellular molecules other than DNA[12]. In general, these secondary agents are primarily reactive oxygen species that induce cytotoxicity through chromosomal aberrations. In addition, Demopoulos et al.[12]

Reprint requests to: Shoichi Inoue
Department of Environmental Medicine and Informatics, Graduate School of Environmental Earth Science, Hokkaido University, Kita-10 Nishi-5, Kita-ku, Sapporo 060-0810, Japan.
FAX: +81(11)717-0629
TEL: +81(11)706-2241

found oxidant-induced sister chromatid exchanges and point mutations to a lesser extent than chromosomal aberrations in the presence of reactive oxygen species. The fact is interesting that hyperbaric oxygen induces chromosomal aberrations in eukaryotic cells because a small amount of reactive oxygen is formed through cellular metabolism[13]. Emerit et al.[14] also showed that O_2^- and H_2O_2 induce DNA breaks and chromosomal aberrations. However, the ability of contaminants in drinking water to generate H_2O_2 and subsequently to induce cellular damage remains unknown.

This study was thus undertaken to determine if contaminants, such as chloroform and trichloroethylene, in drinking water generate H_2O_2 in acute human leukemia (HL 60) cells. We found that chloroform, even at low concentrations of 0.1-1.0 mg/L, stimulated H_2O_2 generation in HL-60 cells sensitized by previously treating with 10 nM retinoic acid (RA) for 12 h.

Materials and Methods

Chemicals

Retinoic acid (RA; Fujisawa, Tokyo, Japan) was dissolved in pure ethanol as a 1 mM stock solution, and aliquots of the solution were kept at -70℃ until use. Solutions of 2', 7'-dichlorofluorescein diacetate (DCFH-DA, Eastman Kodak, Rochester, NY) dissolved in ethanol to 1 mM were kept at 4℃. Mono-poly resolving medium [density 1.115 ± 0.002 (mean ± S.D.)] was purchased from ICN Biomedicals Japan (Tokyo, Japan). Pure chloroform, methylene chloride, trichloroethylene, xylene, benzene, and ethanol were purchased from Kanto Chemical (Tokyo, Japan). Fluorochrome-conjugated monoclonal antibodies: anti-CD13 mouse lgG1 was purchased from Nichirei (Tokyo, Japan); anti-CD14 mouse lgG2a, anti-CD33 mouse lgG1, and anti-CD18 lgG1 were obtained from Coulter (Tokyo, Japan); anti-CD34 mouse lgG1 was purchased from Dako Japan (Kyoto, Japan).

Polymorphonuclear Leukocytes (PMN)

Fresh heparinized venous blood was obtained from normal volunteers (two females and three males; ages, 38 ± 15 years). The PMN were separated with a mono-poly resolving medium by centrifugation at 300 g at 20℃ for 30 min[15]. The purity of the PMN was over 95% as determined by May-Giemsa stain. The purified PMN were washed three times with Ca^{2+} and Mg^{2+} free Dulbecco's phosphate buffered saline (PBS) containing 5 mM glucose and 0.1% gelatin (Sigma, St Louis, MO) (PBSg, pH 7.4) and the PMN were adjusted to 5.5×10^5 cells/mL in PBSg.

HL-60 Cells

The cells were kindly provided by Professor K. Shudo, Faculty of Pharmaceutical Science, University of Tokyo. They were routinely maintained in RPMI 1640 medium (GIBCO, Grand Island, NY) supplemented with 5% (1:1) heat-inactivated fetal bovine serum (GIBCO), and 10 mg gentamicin sulfate (Schering-Plough, Osaka, Japan) (complete medium) at 37℃ in humidified 5% CO_2 atmosphere. A half volume of the culture medium was replaced with fresh complete medium every three days[16].

Treatment with Retinoic Acid (RA)

As testing if organic chemical contaminated drinking water can stimulate H_2O_2 production was expected to be difficult because of their relatively low concentrations, HL-60 cells were sensitized by pretreatment of RA to enhance their H_2O_2 productivity more easily in response to small amounts of chemical contaminants. The HL-60 cells were adjusted to 5.5×10^5 cells/mL (final density after addition of 0.1 mL RA solution: 5×10^5 cells/mL), and 0.9 mL aliquots of the cell suspension were preincubated in the complete medium in a 24-well culture plate (Becton Dickinson, Lincoln Park, NJ) for 24 h. After the preincubation, the cells were exposed to graded concentrations (1 nM to 1 μM) of RA for various durations (2 h to 24 h) at 37℃ in humidified 5% CO_2 atmosphere.

Treatment with Organic Compounds

Trichloroethylene and benzene were dissolved in ethanol and then were diluted 1000-fold in the complete medium to abolish the effect of ethanol. Chloroform and methylene chloride were dissolved in PBS and were diluted 1000-fold in the complete medium. Further dilutions for these chemicals were made using the complete medium. The HL-60 cells were incubated with 10 nM RA for 12 h at 37℃ in humidified 5% CO_2 atmosphere after preincubation for 24 h. The PMN and HL-60 cells were treated with 10 μM DCHF-DA for 15 min before the start of incubation with organic compounds, as described by Yuan et al[17]. The PMN were exposed to chloroform and trichloroethylene for 30 or 60 min. The HL-60 cells were incubated with chloroform, methylene chloride, trichloroethylene, benzene or ethanol at concentrations of 0.01 mg/L to 1 mg/L for 60 min, while incubation with xylene at concentrations of 0.01 mg/L to 10mg/L was for 1h. These organic compounds were tested at concentrations below 16.7 to 100 times more than the upper limit of water quality standards (chloroform, 0.06 mg/L; methylene chloride, 0.02 mg/L; trichloroethylene, 0.03 mg/L; benzene, 0.01 mg/L; xylene, 0.4 mg/L) set by the Japan Ministry of Health and Welfare in 1992.

Flow Cytometric Analysis

Because HL-60 cells are classified as M2 FAB subtypes[18], CD13, CD14, CD18, CD33, and CD34 were used to detect the surface antigens expressed on leukemia cell membranes for immunophenotyping. The CD13 and CD14 subtypes are expressed in acute myelocytic leukemia (MI and M5, respectively)[19,20]; CD18 is expressed in both macrophages and granulocytes[21]; CD33 is expressed in acute promyelocytic leukemia[22] and CD34 is expressed in some bone marrow cells and leukemia[23]. The HL-60 cells were stained directly with fluorochrome-labeled anti-CD13, CD14, CD18, CD33 and CD34 IgG, which were measured using a flow cytometer[24].

Production of H_2O_2 in the PMN and HL-60 cells exposed to each organic compound was measured using a flow cytometer (Epics Elite, Coulter, Miami, FL) with a 488 nm excitation argon ion laser. DCFH-DA is metabolized to a nonpolar and non-fluorescent compound of 2', 7'-dichlorofluorescein (DCFH) in the cells, and intracellular H_2O_2 subsequently converts DCFH to a polar and a highly fluorescent compound of dichlorofluorescein (DCF)[17]. The fluorescence of DCF was recorded through a 530/30 nm band pass filter. Because the intensity of intracellular DCF fluorescence indicates the level of H_2O_2, intracellular H_2O_2 levels were expressed as the mean cellular fluorescence intensity in arbitrary units (A. U.). Fluorescent beads (CaliBRITE Beads, Becton Dickinson) were used to standardize and control the flow cytometer: optimizing alignment, reproducing specific operating

conditions, calibrating intensity scales, comparing sensitivities, and monitoring instrument performance[17].

Confocal Laser Microscopic Observation

Samples of HL-60 cells were divided into two parts: one was used for the flow cytometric analyses and the other was used for confocal laser microscopic observation to confirm intracellular production of H_2O_2 optically. Aliquots (200 μL) of HL-60 cells (5×10^5 cells/mL) similarly exposed to DCFH-DA were placed onto 8-chamber slides (Nunc, Napervilole, IL), and then the slides were cytocentrifuged for 1 min at 400 g. The cells were mounted in a mixed solution of glycerol and 10% PBS containing 1 mg/mL p-phenylenediamine and were observed using a confocal imaging system MARC-500 (Bio-Rad. Microscience Division, Watford, UK) equipped with a computer (NEC, Tokyo, Japan).

Statistical Analysis

Concentration-response relationships of methylene chloride, benzene, xylene, ethanol, chloroform, and trichloroethylene to H_2O_2 production in RA-treated HL-60 cells were analyzed by one-way analysis of variance (ANOVA), where significant level was set at $p<0.05$. Each experiment was repeated at least three times to confirm the reproducibility of the data.

Table 1 Hydrogen peroxide production in PMN exposed to chloroform or trichloroethylene for 30 min or 60 min.

Chemical	Concentration (mg/L)	H_2O_2 production (%, mean ± S.D.) 30 min	60 min
chloroform	0.01	100.5 ± 0.6	99.8 ± 0.8
	0.1	100.3 ± 0.8	99.9 ± 0.9
	1	100.5 ± 0.2	99.5 ± 0.5
Trichloroethylene	0.01	99.4 ± 0.3	99.8 ± 0.1
	0.1	100.3 ± 1.5	99.4 ± 0.4
	1	99.6 ± 0.7	98.4 ± 0.8

Each value represents the mean ± S.D. of separate experiments. Data are expressed as % of H_2O_2 in chemcal-treated PMN taking control as 100%.

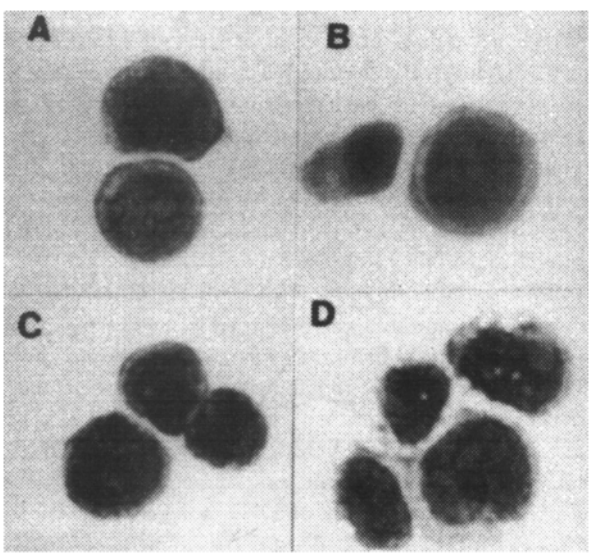

Fig. 1 Cytomorphological change of HL-60 cells after incubation without RA for 1 day (A), with 1 μM RA for 1 day (B), without RA for 3 days (C), and with 1 μM RA for 3 days (D) (May-Giemsa, original magnification ×1000).

Results

Effects of chloroform or trichloroethylene on H_2O_2 production in PMN.

Unlike cytokine-stimulated PMN, untreated PMN hardly produced any H_2O_2 at the resting state, as reported by Yuan et al.[17]. The intracellular H_2O_2 levels in the PMN incubated with chloroform or trichloroethylene alone for 30 or 60 min were similar to the control (Table 1). Chloroform and trichloroethylene did not induce intracellular H_2O_2 production in the PMN.

Morphological changes in HL-60 cells after incubation with RA

The HL-60 cells were stained by the May-Giemsa method after incubating with or without 1 μM RA for one or three days (Figure 1). While the nucleus-to-cytoplasm ratio decreased (Figure 1B) compared with that of untreated HL-60 cells (Figure 1A), the number of cells with cytoplasmic vacuoles (Figure 1D) and slightly constricted nuclei (Figure 1C) increased when HL-60 cells were incubated with 1 μM RA for three days. However, as prominent nucleoli were observed in HL-60 cells treated with RA (Figure 1C, D), the cells remained morphologically at the myeloblast stage. Thus, RA-treated HL-60 cells showed only mild morphological changes, indicating that RA at the concentration of less than 1 μM hardly induced morphological differentiation of HL-60 cells.

Changes in immunophenotype of HL-60 cells after RA treatment

CD13, CD18, and CD 33, in the absence of RA, were positive in HL-60 cells (Figure 2). In contrast, CD14 and CD34 were negative without RA, indicating that HL-60 cells display

Table 2 Relationships in H_2O_2 production between concentration and duration of exposure to retinoic acid (RA).

Concentration (nM)	H_2O_2 production Duration (h) 2	4	8	12	24
1	0.44 ± 0.23	-0.42 ± 0.26	0.19 ± 0.15	0.40 ± 0.15	0.03 ± 0.29
3	0.35 ± 0.21	-0.17 ± 0.25	0.35 ± 0.45	0.67 ± 0.17	0.01 ± 0.17
10	0.36 ± 0.22	0.08 ± 0.24	0.21 ± 0.41	0.47 ± 0.16	0.44 ± 0.42
30	0.63 ± 0.26	0.30 ± 0.24	0.43 ± 0.61	0.80 ± 0.28	0.36 ± 0.26
100	0.58 ± 0.07	0.22 ± 0.36	1.04 ± 1.19	0.98 ± 0.38	0.68 ± 0.42
300	1.03 ± 0.46	0.16 ± 0.20	1.22 ± 1.07	0.98 ± 0.32	0.81 ± 0.62
1000	1.31 ± 0.41	0.54 ± 0.35	1.80 ± 0.90	1.53 ± 0.40	1.43 ± 0.62

Data expressed as the mean ± S.D. in A.U. of 3 different experiments. The A.U. is the H_2O_2 present in HL-60 cells found in the presence of RA minus the amount of H_2O_2 in HL-60 cells found in the absence of RA.

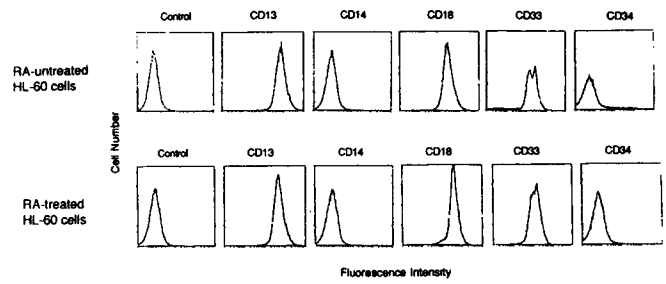

Fig. 2 Expressions of CD13, CD14, CD18, CD33, and CD34 in HL-60 cells in the absence of RA (upper portion) or in the presence of RA (lower portion). Histograms show cell number against fluorescence intensity in arbitrary units.

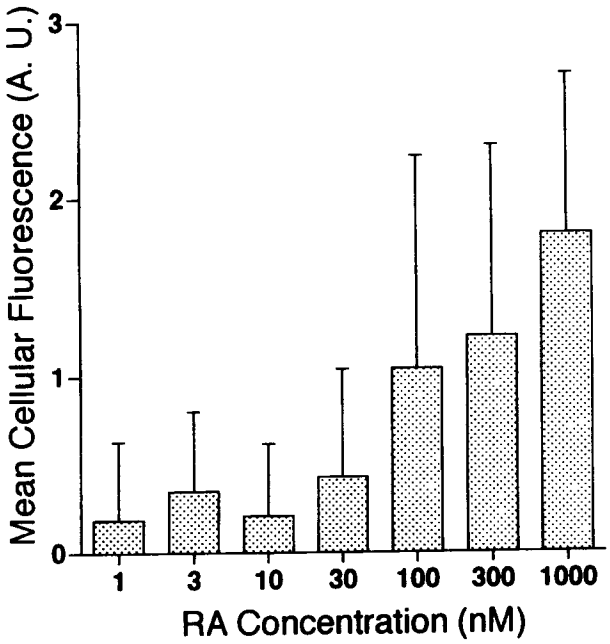

Fig. 3 Relationship between varying retinoic acid (RA) concentrations and H_2O_2 production in HL-60 cells. The HL-60 cells (5×10^5/well) were incubated with various concentrations of RA for 8 h. Data represent the mean ± S.D. of three independent experiments.
A.U. = arbitrary units.

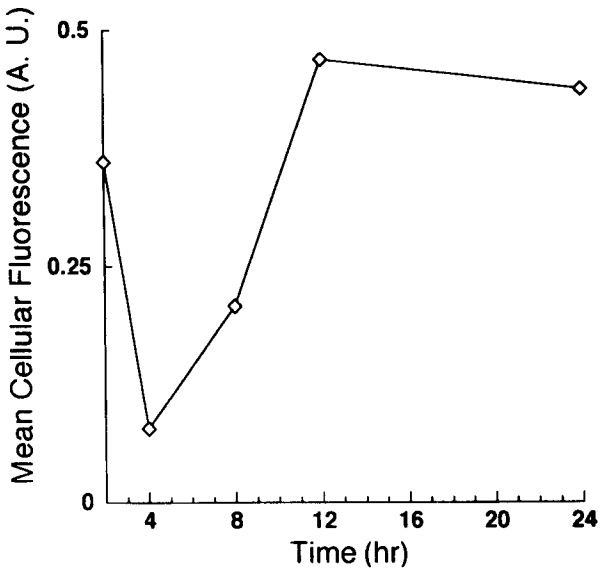

Fig. 4 Time course of retinoic acid (RA) incubation and H_2O_2 production in HL-60 cells. The HL-60 cells (5×10^5/well) were incubated with 10 nM RA for 2-24 h. Data represent the mean of three independent experiments.
A.U. = arbitrary units.

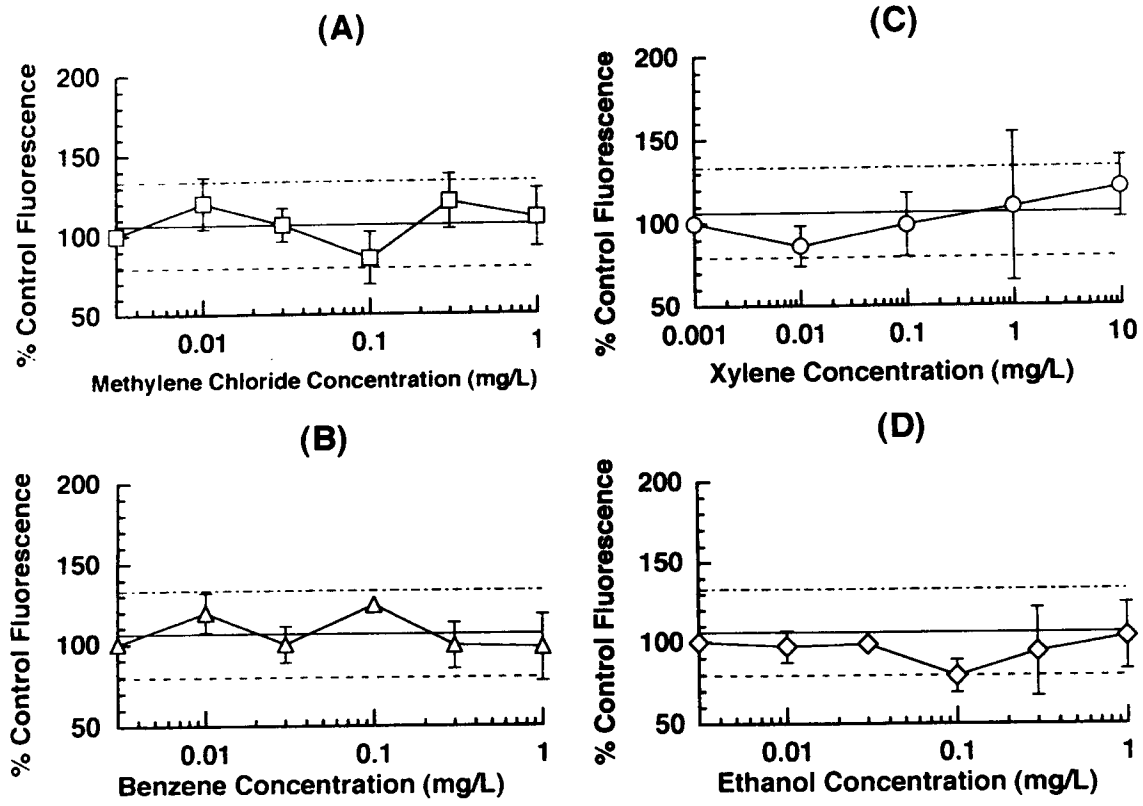

Fig. 5 Effects of methylene chloride, benzene, xylene or ethanol on H_2O_2 production in retinoic acid (RA)-treated HL-60 cells. Data indicate the proportion of control fluorescence of the HL-60 cells sequentially exposed to RA and methylene chloride (A), benzene (B), xylene (C), or ethanol (D). Mean ± 2 σ were indicated by horizontal broken lines. At least three independent experiments were done to obtain the mean value.

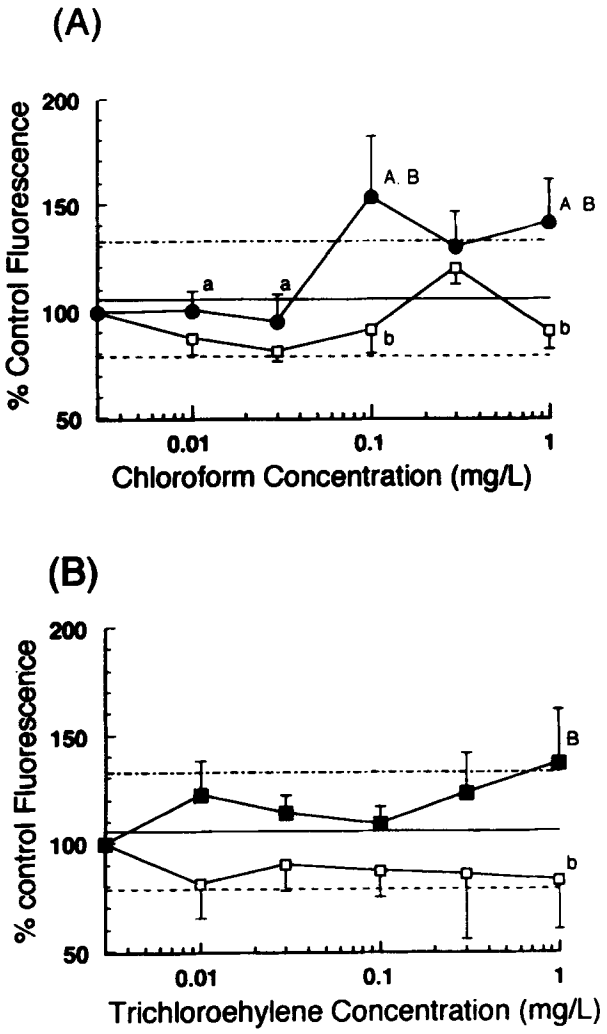

Fig. 6 Effects of chloroform or trichloroethylene on H_2O_2 production in the absence and presence of retinoic acid (RA) by HL-60 cells. Data indicate the proportion of control fluorescence of the cells exposed to chloroform (Panel A) and trichloroethylene (Panel B). The cells were exposed to chloroform for 1 h and to trichloroethylene for 1 h after the incubation with or without 10 nM RA for 12 h. □, untreated HL-60 cells; ●, RA-treated HL-60 cells. Mean ± 2 σ were indicated by horizontal broken lines. The proportion of control fluorescence of the RA-untreated HL-60 cells (□) represents the mean of three independent experiments ± S.D. Significant difference was observed between ^{A} and *, and between ^{B} and ^{b} ($p<0.05$).

characteristics of the myeloblastic subtype, but not of the monocytic form of leukemia. The HL-60 cells treated with 1 μM RA for three days also showed the same immunophenotyping. Thus, the HL-60 cells remained immunophenotypically at the myeloblast stage in the presence or absence of RA.

Relationship between various RA concentrations and H_2O_2 production in HL-60 cells

When HL-60 cells were incubated with 10 nM or more of RA for 8 h, the intracellular H_2O_2 levels increased concentration dependently (Figure 3). Because intracellular H_2O_2 levels in HL-60 cells treated with 10 nM of RA were similar to those in untreated control HL-60 cells, this concentration was deemed optimal to measure chemical-induced H_2O_2 production without an addition of H_2O_2 levels induced by RA treatment.

Relationship between duration of exposure to RA and H_2O_2 production in HL-60 cells

Intracellular H_2O_2 levels in HL-60 cells exposed to 10 nM RA decreased once at 4 h, increased between 4 h and 12 h and then reached a plateau (Figure 4). As the H_2O_2 production reached a plateau after 12 h exposure and as, therefore, we can obtain reproducible data without an addition of H_2O_2 levels induced by RA treatment, the 12 h period was selected as the optimal duration for 10 nM RA incubation.

Table 2 summarizes the relationships in H_2O_2 production between concentration and exposure time of RA. The RA stimulated H_2O_2 production concentration dependently in HL-60 cells. In contrast there was no significant difference in the H_2O_2 production between unstimulated control and cells incubated with 10 nM or less RA.

Effects of methylene chloride, benzene, xylene, and ethanol on H_2O_2 production in RA-treated HL-60 cells

Figure 5 shows the H_2O_2 levels in HL-60 cells previously treated with 10 nM RA for 12 h and then exposed to various concentrations of methylene chloride, benzene, xylene, or ethanol for 1 h. No distinct pattern was observed as values ranged at approximate control levels (106 ± 13.5%, mean ± 2 σ), indicating a lack of significant effect.

Effects of chloroform and trichloroethylene on H_2O_2 production in RA-treated HL-60 cells

When RA-untreated HL-60 cells were exposed to chloroform alone, the H_2O_2 levels in the cells did not increase compared to the control. However, when HL-60 cells sensitized by pretreatment of 10 nM RA for 12 h were exposed to chloroform at concentrations of 0.1 mg/L and more for 1 h, HL-60 cells produced a significant increase in H_2O_2 level compared with those exposed to less than 0.1 mg/L chloroform ($p < 0.05$) (Figure 6A). Furthermore, when HL-60 cells sensitized by pretreatment of RA were exposed to 0.1 and 1 mg/L chloroform, intracellular H_2O_2 levels significantly exceeded those produced in unsensitized HL-60 cells exposed to chloroform alone ($p < 0.05$).

When RA-sensitized HL-60 cells were exposed to 0.1-1 mg/L trichloroethylene, the intracellular H_2O_2 levels apparently increased dose dependently and exceeded the upper range of fluctuations (106 ± 13.5%, mean ± 2 σ) at 1 mg/L (Fig 6B), but the H_2O_2 levels at 1 mg/L did not significantly exceed those exposed to trichloroethylene at 0.1 mg/L or less.

Confocal laser microscopic confirmation of H_2O_2 production in HL-60 cells

When RA-sensitized HL-60 cells were incubated with either methylene chloride, benzene, xylene, or ethanol, the DCF fluorescence was not observed (data not shown). However, the fluorescence was clearly observed in RA-sensitized HL-60 cells exposed to chloroform (Figure 7B) compared to control RA-sensitized HL-60 cells exposed to PBS alone (Figure 7A). Similarly, the fluorescence was observed in RA-sensitized HL-60 cells exposed to trichloroethylene (Figure 7D).

Discussion

The International Agency for Research on Cancer (IARC) and Registry of Toxic Effects of Chemical Substances evaluated the mutagenicity and carcinogenicity of organic contaminants in drinking water[25]. The question arose as to whether these contaminants stimulate intracellular H_2O_2 production. As H_2O_2 has recently been considered as a possible carcinogen[26], whether drinking water contaminants have the ability to induce H_2O_2 generation was examined. The chemical-induced intracellular H_2O_2 levels in PMN or HL-60 cells were measured using a more sensitive flow cytometry method than the simple colorimetric method based on horseradish peroxidase-dependent oxidation of phenol red[27] or on the reduction of nitrobluetetrazolium (NBT)[28].

Chloroform did not increase H_2O_2 production in PMN or in RA-untreated HL-60 cells. However, Eppinger et al.[29] reported that the exposure of HL-60 cells to RA for eight days resulted in differentiation in the granulocytic pathway and an increase in reduction of NBT by stimulating RA-treated HL-60 cells by 12-O-tetradecanoyl-phorbol-13-acetate (PMA). In this study, intracellular H_2O_2 production increased 12 h after incubation with RA, though HL-60 cells did not differentiate into mature cells. This indicates that HL-60 cells treated with RA have the ability to produce H_2O_2 regardless of the presence or absence of morphological maturation. However, the H_2O_2 levels in HL-60 cells morphologically differentiated by RA were less than those produced by PMA stimulation in our previous report[17].

The H_2O_2 production enhanced by chloroform did not increase concentration dependently. The basis for this observation is still unclear. Chloroform was reported to have characteristics similar to radical oxygen species ($\bullet CCl_3$)[30]. On the other hand, the hypothesis of substantial formation of radical intermediates from chloroform can be ruled out[21], and in our study, RA-untreated HL-60 cells and peripheral leukocytes did not show any H_2O_2 production by chloroform stimulation. Therefore, the H_2O_2 production induced by chloroform may not be due to radical intermediates. Furthermore, only a small amount of DCF fluorescence increased concentration dependently when DCFH-DA was exposed directly to H_2O_2 at concentrations of 30 μg/mL or more (data not shown), indicating that chloroform itself does not influence directly the formation of fluorescent DCF. From these reports and our results, H_2O_2 production induced by chloroform seems to be necessary to involve an organelle producing H_2O_2, such as mitochondria, when pretreated with RA. Thus, chloroform may stimulate H_2O_2 production in cells under certain conditions, such as being sensitized by RA to produce H_2O_2 easily in response to stimulants.

Trichloroethylene (0.1-1 mg/L) enhanced intracellular H_2O_2 production dose dependently in HL-60 cells sensitized by RA-pretreatment. The H_2O_2 levels exceeded the upper range of technical error at 1 mg/L, and the DCF fluorescence was observed using a confocal laser microscope. However, the H_2O_2 levels produced in HL-60 cells exposed to trichloroethylene at 1 mg/L did not significantly exceed those exposed at 0.1 mg/L or less. Therefore, we interpret that trichloroethylene remains unproven as a substance that can stimulate intracellular H_2O_2 production in HL-60 cells sensitized by RA-pretreatment, although there is a high possibility that trichloroethylene can stimulate H_2O_2 production at 1 mg/L or more.

The role of H_2O_2 in HL-60 cells sensitized by treating with RA-pretreatment is still unclear. However, many chemical

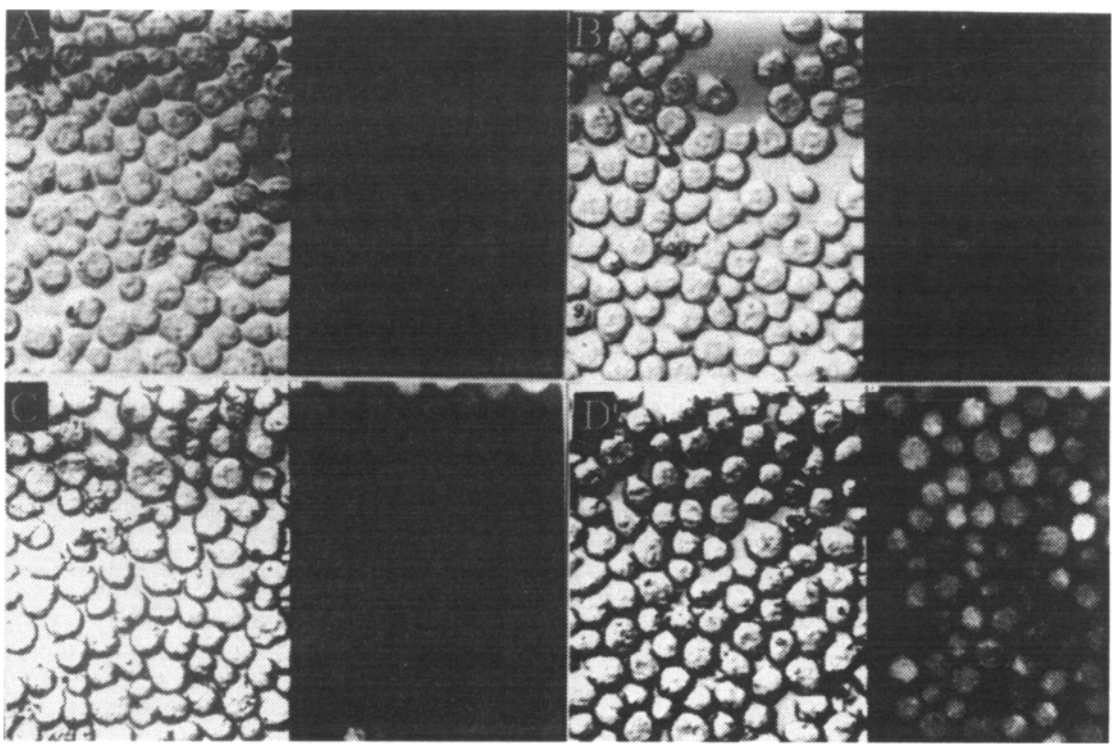

Fig. 7 Confocal laser microscopic observations of HL-60 cells treated with retinoic acid (RA) and then chloroform or trichloroethylene. HL-60 cells were exposed to 1 μg/mL chloroform (Panels A and B) and 1 μg/mL trichloroethylene (Panels C and D) after treated with 10 nM RA for 12 h (Panels B and D) or with PBS alone (Panels A and C). HL-60 cells were observed as described in "Materials and Methods". Each panel shows brightfield image (left) and fluorescence image (right) of HL-60 cells in the same visual field.

carcinogens are thought to cause normal stem cells to change to intermediate stem cells, followed by a change into malignant cells[31]. Thus, stem cells, but not mature cells have a main role in carcinogenesis. As intermediate stem cells are not clearly defined, HL-60 cells sensitized by pretreatment of RA to respond to stimulants easily may possibly have common functions as intermediate stem cells. Our results indicate that chloroform stimulates H_2O_2 production in HL-60 cells sensitized by RA pretreatment. The role of this chemical-induced intracellular H_2O_2 in carcinogenesis is still a field of intensive research.

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