ORIGINAL ARTICLE

Development of a toxicity evaluation system for gaseous compounds using air-liquid interface culture of a human bronchial epithelial cell line, Calu-3

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Abstract

To mimic *in vivo* condition for exposure to gaseous compounds, we employed the Air-Liquid Interface Culture (ALIC) of a human bronchial epithelial cell line, Calu-3, and developed a simple batch-type closed system for direct gas exposure. This system enabled relatively simple cytoxicity evaluation of various gaseous chemicals in an in-vivo mimicking manner. As a preliminary evaluation of the system developed, we tested the toxicity expression of Calu-3 to benzene, tetrachloroethylene and acetone gases in terms of lactate dehydrogenase (LDH) release during 48 hours of the loading. The toxicity in ALIC exposure was higher than that in conventional exposure in the liquid phase. The reason was largely explained by numerical estimation that chemical concentrations exactly on the cell surface in the liquid culture is lower in such acute phase exposure than that in ALIC culture, in the cause of the diffusion process of molecules in the surface liquid layer. These results indicate that basic concept of the combination of ALIC of lung cells and a simple batch-type closed system is promising as a cytotoxicity test of wide ranges of gaseous compounds or samples.

***Keywords**: Cytotoxicity, Exposure system, Gaseous compound, Air-liquid interface culture (ALIC), Calu-3 cell,*

Introduction

Because of the difficulties in establishing simple *in vitro* gas exposure systems, animal experiments are still the main toxicity tests for gaseous compounds or samples. To overcome this situation, several *in vitro* systems with respiratory cells have been proposed. Conventional studies used static culture dishes (Patel *et al.*, 1990), dishes on tilting platforms (Guerrero *et al.*, 1979; Nikula *et al.*, 1990; Dumler *et al.*, 1994), rotating flask-cells (Pace *et*

al., 1969; Bank *et al.*, 1990), roller bottles (Bolton *et al.*, 1982), bubbling gas through cell suspensions (Konings, 1986), gas-permeable membranes with a culture medium above and a gas flow beneath (Alink *et al.*, 1980; Cheek *et al.*, 1988). Direct gas exposure has also been achieved by using a monolayer culture cells in an inverted state, but the duration of exposure was 10-60 min (Tsuda *et al.*, 1981; Tu *et al.*, 1995). In summary, those methods have not achieved both stable cell culture and *in vivo*-mimicking direct gas exposure via a liquid layer less than 10 μm thick.

Recently, a method known as the air-liquid interface culture (ALIC) of bronchial epithelial cells has been developed (Whittcutt *et al.*, 1988; Johnson *et al.*, 1993). ALIC enables cells to contact gases directly with the apical sides while culture medium is supplied from the basal side through semi-permeable membrane. By using this ALIC technique, a number of human lung epithelial cell lines grown at the air-liquid interface are available with higher levels of differentiation (Whittcutt *et al.*, 1988; Johnson *et al.*, 1993; Shen *et al.*, 1994). Among them, a human bronchial epithelial cell line, Calu-3, is a well-characterized one (Shen *et al.*, 1994). Although there is limitations such as incomplete ciliation, the cells cultured at the air-liquid interface have often been used as an in vitro models for the human airway epithelium (Foster *et al.*, 2000; Florea *et al.*, 2003), because they resemble native tissue and primary cultures in that they form tight junctions as determined by electron microscopy, contain mucous secretory granules, and show active transepithelial secretion of Cl (Shen *et al.*, 1994).

In previously-developed gas exposure systems using lung epithelial cells in ALIC, usually it was necessary to supply the exposure chamber with a continuous flow of samples gases. This is simply because the targets of cytotoxicity evaluation were gases having relatively short life times, such as acid sulfate aerosol (Chen *et al.*, 1993) or diesel motor exhaust (Knebel *et al.*, 2002). However, when we think about cytotoxicity assessment of much larger ranges of gas samples such as those in environments or from industries, uses of simple batch-type exposure systems are helpful.

Therefore, in this study, we developed a simple batch-type closed exposure system for evaluating the toxicity of gaseous compounds. Using ALIC of Calu-3 cells, we tested the cytotoxicity of some gaseous volatile organic compounds known as contaminants of the natural or occupational environments. Additionally, we compared the toxicity in ALIC and liquid phase exposures, and investigated the effects of the diffusion of these chemicals in a liquid layer over the cell layer.

Materials and methods

Cell culture and medium

Calu-3 cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). The cells were suspended in a 1:1 mixture of Dulbecco's modified Eagle medium (DMEM) and Ham's F-12 medium (GIBCO, Life Technol., Grand Island, NY, USA), supplemented with 10% fetal bovine serum (Filtron, Altona, Australia), 20 mM hydroxyethylpiperazine-N'2-ethanesulfonic acid (HEPES, Dojindo, Kumamoto, Japan), penicillin (100 units/ml) (Wako, Osaka, Japan), streptomycin (100 μg/ml) (Wako), and amphotericin B (1.0 μg/ml) (Sigma Chemical, St. Louis, MO, USA). During the exposure experiment, cells were maintained in the above medium supplemented with 5% fetal bovine serum. The cell lines were subcultivated using 0.25% trypsin and 0.02% EDTA in phosphate-buffered saline (PBS).

Formation of Calu-3 cell layer on a membrane support

After being coated with Vitrogen plating medium containing DMEM (GIBCO), bovine fibronectin (10 μg/ml; Yagai Corp., Yamagata, Japan), 1% Vitrogen collagen (Cohesion Technol. Inc., Palo Alto, CA, USA), and bovine serum albumin (10μg /ml; Sigma Chemical) (Loffing et al., 1998), the Calu-3 cells were seeded at a density of 1×10^5 cells/cm^2 onto a polyester membrane culture insert with a culture surface area of 1.0 cm^2 and a pore size of 0.4 μm (Transwell 3460, Coaster, Cambridge, MA, USA). The confluence of the cell sheet were detectable when the trans-epithelium electrical resistance (TEER) reflecting function of tight junction (Widdicombe, 1990) was measured with a Millicell-ERS (Millipore Corp., Bedford, MA, USA).

Gas exposure system

The experimental system used for gas exposure is shown in Fig. 1. It was constructed of glass to withstand the effects of organic solvents. The dimensions of the system are 150 × φ150 mm, and the inner volume is about 1000 ml. An Omnipore Membrane Filter made of polyester with a culture

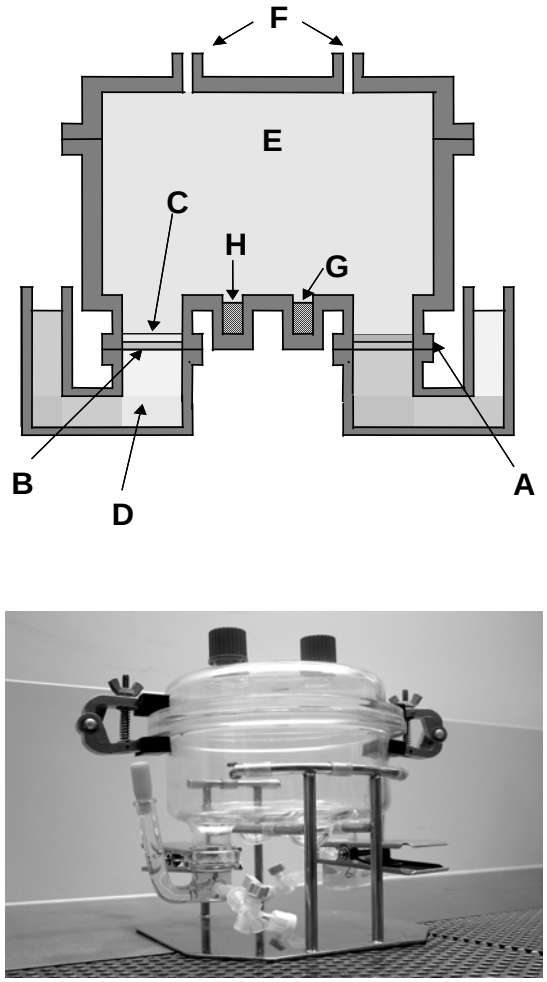

Figure 1. Schematic representation and photograph of the *in vitro* experimental system developed for gas exposure to cells in air-liquid interface culture (ALIC). A, Filter holder; B, Membrane filter; C, Human bronchial cells, Calu-3; D, Culture medium; E, Closed gas chamber; F, Gas sampling hole; G, Chemical substance; H, Water for humidification. This system accommodates two cell-loaded membranes under the same exposure and culture condition.

surface area of 3.0 cm^2 and a pore size of 0.4 μm (Millipore Corp.) was sterilized by ethylene oxide gas and placed in the filter holder. The Calu-3 cells were plated at a density of 1×10^5 cells/cm^2 onto the membrane filter after being coated with Vitrogen plating medium. The culture medium was added to only the basal side; no medium was added to the mucosal surface.

The gas chamber was hermetically sealed, and the internal gas concentration was quantitatively measured with a gas chromatograph (GC-FID, Shimadzu, Osaka, Japan). A certain amount of chemicals (benzene or tetrachloroethylene or acetone) in liquid phase (5-1000 μl) was placed into a depression at the bottom of the vessel. Changes in the internal gas concentration were checked in advance, as shown in Fig. 2. Although it decreased slightly, the concentration was maintained at more than 70% of its initial level for two days. As a means of humidity control, water was placed into a second depression in the vessel. The exposure time was one or two days.

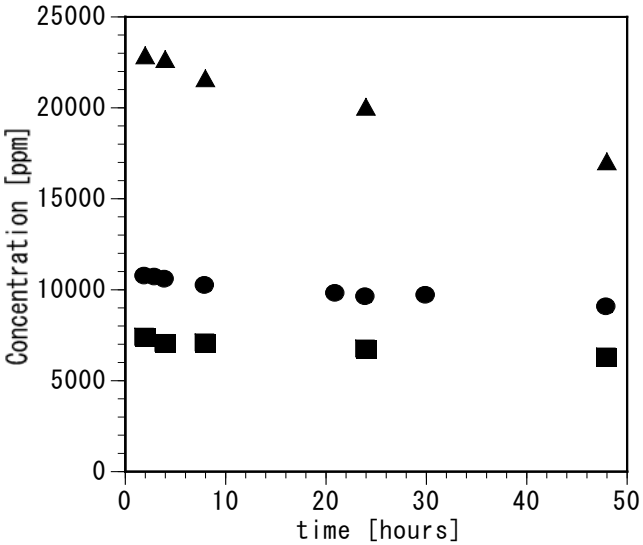

Figure 2. Change in the gaseous concentrations of benzene (●), tetrachloroethylene (■) and acetone (▲) in the exposure system developed. The initial concentrations are 10,700 ppm for benzene, 7,300 ppm for tetrachloroethylene and 22,800 ppm for acetone.

Cytotoxicity Assays

We measured lactate dehydrogenase (LDH) release into the culture medium using a reagent kit (LDH-Cytotoxic Test, Wako) (Korzeniewski *et al.*, 1983). LDH release has been used as an indicator of cytotoxicity of gaseous compounds to cultured lung epithelial cells in a number of studies (Dumler *et al.*, 1994; Tu *et al.*, 1995). The released LDH was measured from the culture medium of the exposed cells (released $LDH_{exposure}$). After exposure, the remaining cells were soaked in PBS containing 0.2% Tween20 and were destroyed cytomembrane. After two hours of incubation at

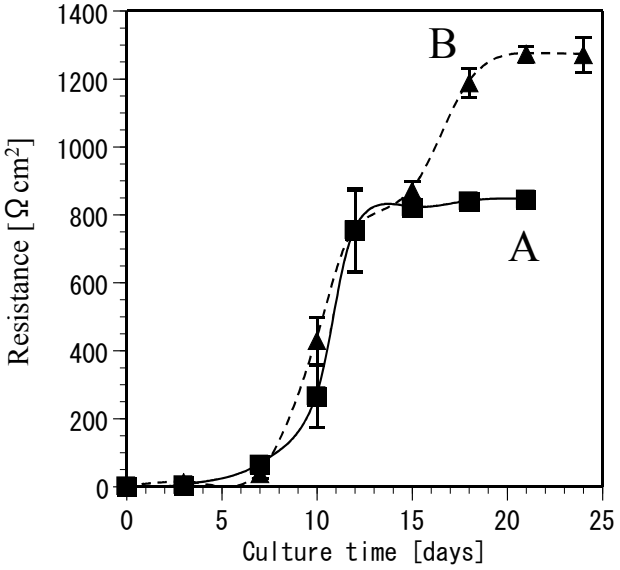

Figure 3. Changes in the trans-epithelium electrical resistance (TEER) of Calu-3 cells. The data was obtained from cultures in the liquid phase (■) and from cultures where the apical medium was removed after 14 days of cultivation (▲). All data subtracted the value of blank (resistance of the membrane and the culture medium), 90 Ωcm^2. All plots in the figure were the mean ± S.D. of four wells from two independent experiments.

37℃, the remaining LDH (remaining $LDH_{exposure}$) was obtained from the PBS. The value of both LDH measurements subtracted that of LDH of a fresh culture medium and from PBS itself, respectively. We defined cell viability as:

$$Viability = \frac{remainingLDH_{exposure}}{releasedLDH_{exposure} + remainingLDH_{exposure}}$$

Results

Development of Calu-3 cell layer during ALIC

To assess the time course formation of the cell layer, the change in the trans-epithelium electrical resistance (TEER) was first examined at the culture insert. The results are shown in Fig. 3. The medium was added to both the apical and basal sides. As shown in curve A, the TEER increased rapidly with an increased cultivation time and reached a steady state around at about 800 Ωcm^2 on day 12. Curve B shows the results from an experiment in which the apical medium was removed after day 14 of cultivation. In this situation, culture medium of basal side didn't leak to mucosal side, ALIC condition was made possible. The resistance increased again and a resistance of 1300 Ωcm^2 was obtained three days after the removal of the apical medium.

Response of cells to benzene, tetrachloroethylene and acetone exposure

We further investigated the acute toxicity of benzene, tetrachloroethylene and acetone to the cells for periods of one or two days. The results are shown in Fig. 4-a, b, c. Under the control condition and in low concentrations, cell viability in terms of LDH release remained near 100 % on all of three chemical gases. However, the viability was reduced with increased gas concentrations, as compared with the value obtained for cultures with

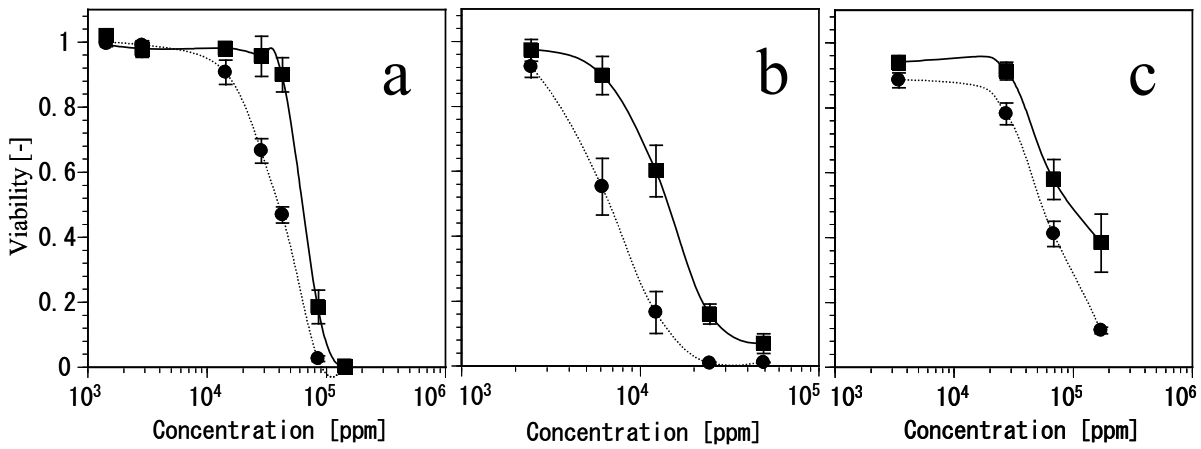

Figure 4. Dose-response curves of Calu-3 cells cultured for one (■) or two (●) days at the air-liquid interface when loaded with benzene (a), tetrachloroethylene (b) and acetone (c) in a gas phase. All plots in the figure were the mean ± S.D. of four wells from two independent experiments.

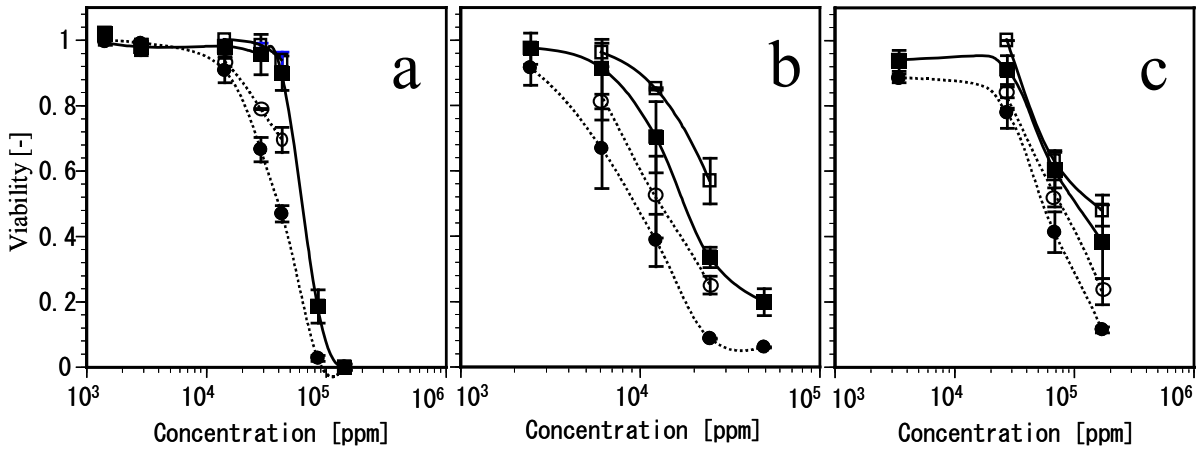

Figure 5. Comparison of cytotoxicity between ALIC exposure and in-liquid phase exposure when loaded with benzene (a), tetrachloroethylene (b) and acetone (c) gases. The exposure times were one day (■) and two days (●) under the ALIC condition, and one day (□) and two days (○) under the liquid-phase condition. All plots in the figure were the mean ± S.D. of four wells from two independent experiments.

no exposure (control condition). Therefore, the developed system successfully enabled the evaluation of acute toxicity in *in vivo*-mimicking exposure conditions.

Effects of a liquid layer on benzene, tetrachloroethylene and acetone toxicity

To clarify the importance of direct exposure, 600 µl of culture medium (the culture medium layer was 2-mm thick, which is the standard depth used in most cell cultures) was added to the surface of the cells prior to exposure, and compared with ALIC exposure. The cells of both types of exposure were differentiated by cultivating at the air-liquid interface in advance.

The results are shown in Fig. 5-a, b, c. About all of three chemicals, when the medium was added to the surface of the cells, the damage caused by these chemicals was reduced. It is apparent that the presence of the surface liquid layer inhibited the expression of toxicity, demonstrating that direct exposure mimicking the *in vivo* situation is significant in testing toxicity of gaseous compounds *in vitro*.

Effects of diffusion in a surface liquid layer

The surface liquid layer of cells inhibited gas exposure because it strongly inhibited the transfer of compounds. Therefore, the effect of diffusion of benzene, tetrachloroethylene and acetone molecules in the surface liquid layer on cytotoxicity was evaluated by estimating the time dependency of chemical concentrations at exactly the cell layer surface using the following simple diffusion equation.

$$\frac{\partial C}{\partial t} = D_m \frac{\partial^2 C}{\partial z^2} \qquad (1)$$

where D_m is the diffusion coefficient and z is vertical distance from the surface of the liquid layer. Assuming the initial conditions to be t=0, C=0 at Z>0, and the boundary conditions to be t>0, C=1 at Z=0, and t>0 C=0 at Z=∞, the benzene concentration in the liquid is written as Eq. 2.

$$C(z,t) = 1 - erf\,(x) \qquad (2)$$

where

$$erf\,x = \frac{2}{\sqrt{\pi}} \int_0^x e^{-x^2} dx \qquad (3)$$

and

$$x = \frac{z}{\sqrt{4 D_m t}} \qquad (4)$$

The value of diffusion coefficient, D_m, was 1.01×10^{-5} cm^2/s (benzene), 9.06×10^{-6} cm^2/s (tetrachloroethylene), 1.19×10^{-5} cm^2/s (acetone) at 37℃. (Wilke *et al.*, 1955).

The predicted time dependency of these chemical concentrations at the surface of the cell layer is shown in Fig. 6-a, b, c. The concentration in the liquid phase condition, where the thickness of the liquid layer was 2 mm, was apparently lowered when compared with that of ALIC, where the thickness of the liquid layer was 10 µm. This

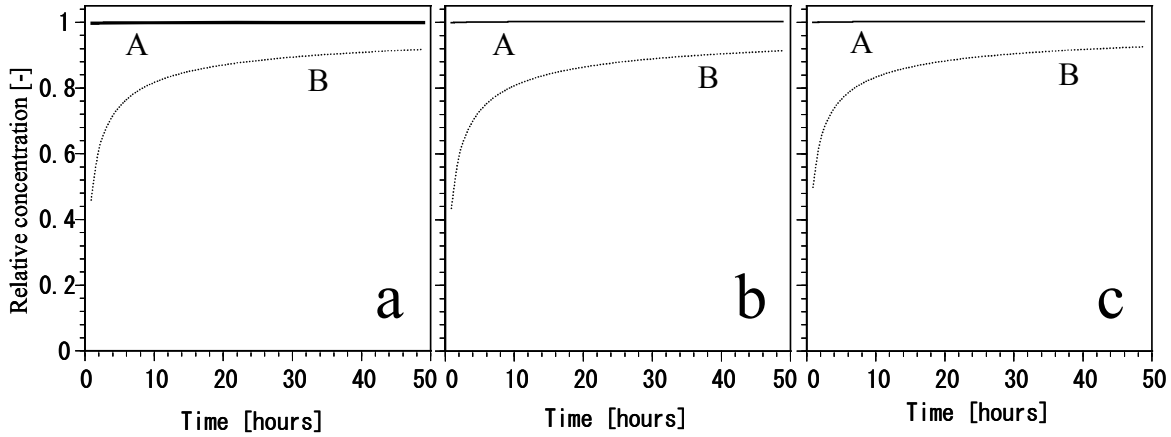

Figure 6. Calculated time dependency of benzene (a), tetrachloroethylene (b) and acetone (c) concentrations at the surface of the cell layer when we considered the effect of diffusion of these molecules in the surface liquid layer on the cells. The solid line (curve A) indicates the ALIC condition (the thickness of the liquid layer is 10 μm) and the dotted line (curve B) indicates the liquid-phase condition (the thickness of the liquid layer is 2 mm).

result clearly demonstrated the suitability of ALIC in estimating the toxicity of gaseous compounds.

Total exposure

It is obvious that exposure strength depends on both the concentration of the test compounds and the exposure time. As the simplest assumption, the time-integrated concentration, c×t, has been used in estimating total exposure (de Vries, 1996). Therefore, the total exposure from our ALIC exposure data and exposure through a surface liquid layer were compared in terms of cell viability based on the above concept. With regard to exposure through the surface liquid layer, the value of c×t was obtained from the area, as shown in Fig. 6. The ratio of the area in the liquid culture to that in ALIC was 0.79 (benzene), 0.78 (tetrachloroethylene), 0.81 (acetone) under the concentration curves for one day of exposure and 0.85 (benzene), 0.84 (tetrachloroethylene), 0.86 (acetone) for two days of exposure.

The results of the comparison are shown in Fig. 7-a, b, c. The data for the one- and two-day assays for each compound under ALIC exposure are shown on one curve respectively. In addition, the data from exposure through the surface liquid layer in which the diffusion process in the liquid layer was considered are also shown on the same curve. These results demonstrate that toxicity is strongly affected by the diffusion of compounds in a surface liquid layer. Furthermore, acute toxicity can be adequately extrapolated through the concept

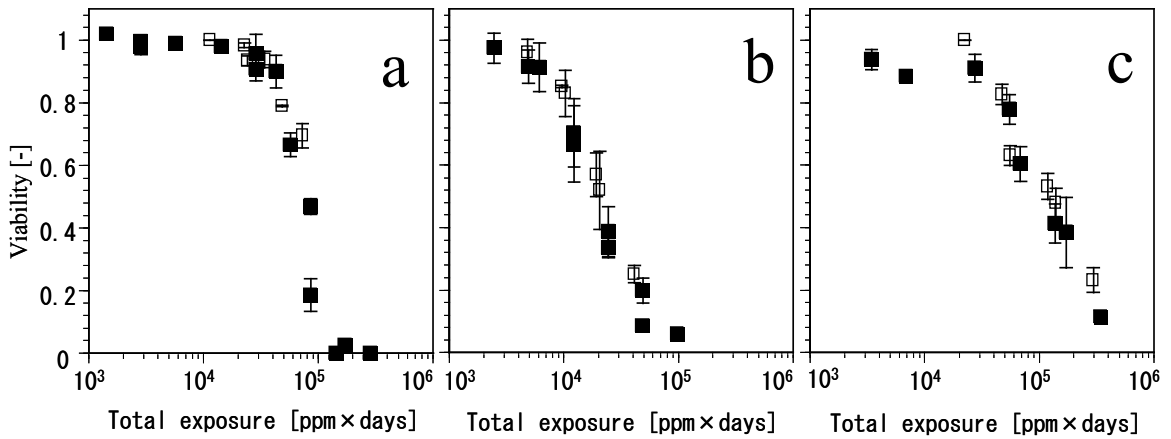

Figure 7. Relationship between total exposure and cell viability when loaded with benzene (a), tetrachloroethylene (b) and acetone (c) gases. The plots indicate the calculated data under the ALIC condition (■) and under the liquid-phase condition (□).

of total exposure.

Discussion

In this study, we developed a simple closed chamber-based gas exposure system where lung epithelial cells can stable maintained in ALIC. This system has significant advantages over previously described exposure systems for cytotoxicity of gaseous compounds, in that mimicking the *in vivo* exposure condition where lung epithelia cells are in close and semi-direct contact with gas phases, allowing cells to be stably exposed for several days, and being capable of convenient evaluation without using an extensively large volume gas chamber.

As a first application of this exposure system, we employed the Calu-3 cell line derived from human bronchial epithelium. Shen *et al.* (1994) and Loffing *et al.* (1998) indicated that the cells cultured at the air-liquid interface appeared more differentiated than in liquid condition, from the facts not only that the cells contained mucous secretory granules but also that the TEER became higher by the formation of tight junction and formation multilayers upon the commencement of ALIC. These results demonstrate that the changes of TEER value in response to ALIC can be a simple and good indication for more differentiated phases of the cells *in vitro*. Therefore, to better mimic the exposure to actual bronchial epithelium, we performed the gas exposure studies after confirming a further increase in TEER values in ALIC, as can be seen in Fig. 5.

In this study, we evaluated toxicity of three typical volatile organic compounds in their pure forms and the toxicity was able to be explained the time integral of the concentrations, $c \times t$, at the surface of the cell layer. However, as opposed to the exposure to such pure gaseous compounds, it is extremely difficult for compound mixtures to evaluate their toxicity in the same manner, because we need some physical properties like diffusion coefficients of each compound contained in the mixtures. Therefore, this developed system is superior as a simple testing system for cytotoxicity of gaseous samples such as environmental or occupational ones, because we can directly evaluate the overall toxicity of such mixtures without knowing such properties of contained compounds.

It is well known that the short-term acute exposure of volatile organic chemicals, such as the three chemicals used in our experiments, mainly cause neurological malfunctions or pulmonary irritation (WHO, 1993; Gangolli, 1999). However, no significant damage has been reported in the lung epithelium in the concentration ranges where such lethal damage occurs (WHO, 1993; Gangolli, 1999). In our experiment, when we compare our data with some known data in animal experiments, for example LC_{50} (benzene; 5,000 ppm for eight hours to mouse (WHO, 1993)), the value of *in vitro* LC_{50} in our experiment (benzene; 60,000 ppm for 24 hours to Calu-3 cells, Fig. 4-a) is relatively large. According to a previous study, biotransformation of benzene appears to contribute to the *in vivo* lethal toxicity (Gangolli, 1999). However, such biotransformation is presumed to occur mainly in the liver (Gangolli, 1999), and the Calu-3 cells has low levels of cytochrome P450s (Foster *et al.*, 2000). Therefore, the death of animals does not depend on the death of the bronchial tissue but depend on damage to other organs such as nerve tissues partly after biotransformation in the liver.

As a future application of the developed exposure system, it can also be used in simply estimating the cytotoxicity to various epithelial tissues at air-liquid interfaces, such as alveolar, nasal or skin tissues. Therefore, we would clarify such differences in sensitivity among different tissues. In addition, through *in vitro* studies using the developed simple gas exposure system and other conventional cytotoxicity tests based on liquid culture, we will be able to obtain a better knowledge of whole toxicity mechanisms of gaseous compounds to humans.

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