ISOLATION AND CHARACTERIZATION OF RAT PRIMARY LUNG CELLS[1]

DANIELA S. BUNDSCHUH,[2] STEFAN UHLIG, MARCEL LEIST, ACHIM SAUER, and ALBRECHT WENDEL

Biochemical Pharmacology, Faculty of Biology, University of Konstanz, D-78434, Konstanz, Germany

Summary

Lung cell culture may be useful as an *in vitro* alternative to study the susceptibility of the lung to various toxic agents. Lungs from female Wistar rats were enzymatically digested by recirculating perfusion through the pulmonary artery with a sequence of solutions containing deoxyribonuclease, chymopapain, pronase, collagenase, and elastase. Lung tissue was microdissected and resuspended and the cells obtained were washed by centrifugation. By this isolation method, 2×10^8 cells per rat lung were obtained with an average viability of 97%. Lung cells cultured in medium containing antibiotics and serum maintained a viability of > 70% for 5 d. Rat primary lung cells were exposed to various toxic agents and their viability was assessed by formazan production capacity after 18 h of incubation. Compared to rat and mouse hepatocyte cultures (EC_{50} = 5.8 m*M*), rat primary lung cells were much more susceptible to hydrogen peroxide (EC_{50} = 0.6 m*M*). All cell types were equally sensitive to the more potent toxicant *tert*-butylhydroperoxide (EC_{50} = 0.1 m*M*). Paraquat was more toxic to lung cells (EC_{50} = 0.03 m*M*) than to rat (EC_{50} = 2.8 m*M*) and mouse (EC_{50} = 0.2 m*M*) hepatocytes. In contrast, rat lung cells were less sensitive to sodium nitroprusside (EC_{50} = 2.6 m*M*) compared to rat (EC_{50} = 0.2 m*M*) and mouse (EC_{50} = 0.03 m*M*) hepatocytes. Nitrofurantoin and menadione (at EC_{50} = 0.04 m*M* and 0.006 m*M*, respectively) were more toxic to rat lung and liver cells than to murine hepatocytes (EC_{50} = 0.2 m*M* and 0.04 m*M*, respectively). Our findings demonstrate the applicability of this rat primary lung cell culture for studying the effects of lung toxicants.

Key words: lung digestion; primary lung cells; primary hepatocytes; toxicity studies.

Introduction

The cellular consequences of endogenous or exogenous influences on pulmonary metabolism are of prime diagnostic and therapeutic interest. As an alternative to the use of experimental animals, cell cultures greatly facilitate studies on these issues. In culture, experimental conditions can be easily controlled and specific cell types as well as cell–cell interactions can be studied. Commonly, immortalized cell lines or primary cells obtained by either bronchoalveolar lavage or digestion of pulmonary tissue provide the material for culturing pulmonary cells. These approaches have limitations, in that immortalized cell lines may not represent the original tissue cell type and mainly alveolar macrophages are isolated by bronchoalveolar lavage. As an alternative method, enzymatic digestion of whole lung tissue allows harvest of pulmonary cell types that may retain many properties of the original cell type during culture.

Several different techniques have been used to isolate the various types of lung cells from rat or other mammalian species. These include mechanical disaggregation of lung tissue by mincing (Van Overveld et al., 1987; Lavnikova et al., 1993) or mashing (Lavnikova et al., 1993) and enzymatic digestion by instillation and/or immersion using trypsin (Kikkawa and Yoneda, 1974; Brown and Longmore, 1986), elastase (Dobbs and Mason, 1979; Sikpi et al., 1986; Mangum et al., 1990), collagenase (Hinz and Syverton, 1959; Douglas and Kaighn, 1974), or combinations of these and other enzymes [i.e., deoxyribonuclease, pronase, catalase (Gould et al., 1972; Phillips, 1972; Finkelstein et al., 1983; Van Overveld et al., 1987; Berk et al., 1991; Jassal et al., 1991)]. Efforts to isolate lung cells from rats have been directed mainly towards obtaining cells of a single cell type [i.e., type II pneumocytes (Kikkawa and Yoneda, 1974; Douglas and Teel, 1976; Mason et al., 1977; Dobbs and Mason, 1979; Goodman et al., 1983; Post et al., 1984; Brown and Longmore, 1986; Dobbs et al., 1986; Batenburg et al., 1988; Ma et al., 1989), interstitial macrophages (Lavnikova et al., 1993; Berk et al., 1991), or mast cells (Van Overveld et al., 1987)].

All previously reported methods for preparation of pneumocyte cultures were based on digestion of minced pulmonary tissue, suitable for preparation of only a limited number of cell types. However, because lung tissue consists of a heterogeneous population of about 40 different cell types (Sorokin, 1970), and no single cell type seems to predominate in the organ, we were interested in a primary lung cell culture maintaining cellular heterogeneity. In order to achieve this goal, in lung tissue digestive enzymes must reach a large surface area. One solution is to perfuse digestive enzymes through the pulmonary artery rather than to digest mechanically minced lung tissue.

The objective of this study was to prepare primary rat lung cell culture containing the major pulmonary cell populations and to study their functional response to various toxic agents in comparison to rodent hepatocytes.

---

[1] Parts of the study had been presented orally at the meeting of the German Society of Toxicology and Pharmacology in Mainz (FRG), March 15–17, 1994.

[2] To whom correspondence should be addressed at Faculty of Biology, Biochemical Pharmacology, University of Konstanz, POB 5560 M668, D-78434 Konstanz, Germany.

Materials and Methods

Animals

Male, specific pathogen-free BALB/c mice (25 g; from the internal breeding house, University of Konstanz, Germany) and female, specific pathogen-free Wistar rats (200–250 g; Zentralinstitut für Versuchstiere, Hannover, Germany) were kept under controlled conditions (22° C, 55% humidity, 12 h day/night rhythm) on a standard laboratory chow (Altromin 1313/Altromin 1314). Animals received humane care in adherence to the NIH guidelines, as well as to legal requirements in Germany.

Isolation of Rat Lung Cells

Method I: Instillation and immersion. Female wistar rats were anesthetized by intraperitoneal injection of 60 mg/kg Nembutal® (Sanofi Winthrop, München, Germany) and were exsanguinated. The lungs were perfused through the pulmonary artery with 100 ml of warm (38° C) RPMI 1640 (Biochrom, Berlin, Germany) containing 2% albumin (Sigma, München, Germany), 100 IU/ml penicillin and 100 μg/ml streptomycin (Biochrom). When the lungs were completely free of blood, the entire organs were excised and enzyme solution I [0.5 mg/ml deoxyribonuclease I (E.C. 3.1.21.1; Sigma, München, Germany), 0.5 mg/ml chymopapain (E.C. 3.4.22.6; Sigma), and 2 mg/ml pronase (Boehringer Mannheim, Mannheim, Germany) in Tyrode's buffer (Van Overveld et al., 1987)] was instilled via the trachea. After ligation of the trachea, the lung was immersed in enzyme solution I and incubated for 18 min in a warm (37° C) shaking water bath (130 rpm), followed by removal of the trachea and major bronchi and microdissection of the lungs (on ice) into ~ 500 μm slices. The tissue slices were incubated twice in enzyme solution II [0.5 mg/ml deoxyribonuclease I, 1 mg/ml collagenase H (E.C. 3.4.24.3; Boehringer Mannheim) and 0.1 U/ml elastase (E.C. 3.4.21.36; Sigma) in Tyrode's buffer] for 18 min in a shaking water bath (130 rpm, 37° C). Cells released at each stage were separated from the tissue by filtration (100 μm/60 μm mesh filters) and were pooled. After the cells had been washed twice in RPMI 1640 (370 g, 4° C, 10 min), cell yield and viability of isolated cells were determined.

Method II: Recirculating perfusion. After anesthesia (60 mg/kg Nembutal®) of female Wistar rats, and subsequent tracheotomy and heparin injection (500 IU into the right ventricle), the lungs were excised and perfused with 100 ml of warm (38° C) RPMI 1640 containing 2% albumin and antibiotics. Then, the lungs were perfused with 100 ml enzyme solution III (0.5 mg/ml deoxyribonuclease, 0.5 mg/ml chymopapain, and 2 mg/ml pronase in RPMI 1640 containing 2% albumin and antibiotics) followed by perfusion with 100 ml of enzyme solution IV (0.5 mg/ml deoxyribonuclease I, 1 mg/ml collagenase H, and 0.1 U/ml elastase in RPMI 1640 containing 2% albumin and antibiotics). During surgery and perfusion, lungs were ventilated by positive pressure (respiratory rate: 80 breaths/min, tidal volume: 1.5 ml) to improve the viability of lung cells and the removal of blood and to accelerate the digestion. The perfusates from perfusion with enzyme solution III and IV were collected. Subsequently, both perfusates were pooled and used for recirculating perfusion of the lung until the organ was completely edematous and the lung tissue was digested. After removal of heart and trachea, the lungs were divided into five lobes that were cut into ~ 500 μm slices. In order to release cells from the tissue matrix, these tissue slices were resuspended (20–30 times, soaking and blowing out) with 10-ml plastic pipettes. Free cells released at this stage were separated from the tissue by filtration (100 μm/60 μm mesh filters) and were pooled. After washing of cells in RPMI 1640 (370 g, 4° C, 10 min), cell yield and viability of isolated cells were determined.

Isolation of Hepatocytes

Murine hepatocytes. Hepatocytes from male BALB/c mice were prepared by the two-step collagenase perfusion method of Seglen (1976) as modified by Klaunig et al. (1981a; 1981b). The cell preparation contained mainly hepatocytes and about 5% Kupffer cells as determined by unspecific esterase staining and phagocytosis of fluorescein-labeled latex (Ø 0.75 μm; Polyscience Inc., Eppelheim, Germany).

Rat hepatocytes. Hepatocytes from female Wistar rats were isolated according to the method of Seglen (1976) as modified by Hartung and Wendel (1991). After several washing steps and differential centrifugation, hepatocyte preparations contained ~ 3% Kupffer cells as determined by unspecific esterase staining and phagocytosis of fluorescein-labeled latex.

Primary Culture of Lung Cells and Hepatocytes

All cells were incubated at 37° C and 5% CO_2.

Lung cell culture. For determination of viability at various time points after isolation, 1.25×10^5 lung cells were plated in 250 μl RPMI 1640 containing 10% fetal bovine serum (FBS) and antibiotics in 24-well plates. Viability of lung cells was determined by LDH (lactate dehydrogenase; E.C. 1.1.1.27) release into supernatants or by trypan blue exclusion at the time points indicated above. For the later time points, medium was changed every 48 h.

In order to adapt the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide] assay for rat lung cells, different cell numbers (0.5, 1, 2, 4 $\times 10^6$ cells/ml), MTT concentrations (0.5, 1 mg/ml), incubation periods with MTT (0.5, 1, 2, 3, 4 h), sizes of culture plates (6-, 24-, 96-well plates; Greiner, Frickenhausen, Germany), and different volumes of cell suspension/well (100, 150, 200 μl/well) were tested. The following conditions were found to be optimum: 4×10^5 lung cells were plated in 200 μl RPMI with serum and antibiotics in 96-well plates. Cells were incubated with 1 mg/ml MTT for 4 h at 37° C and lysed with 30 μl HCl/SDS (3 m*M*/3% final concentration) for 16 h at 37° C.

For toxicity studies, 4×10^5 lung cells were plated in 200 μl RPMI 1640 supplemented with 10% FBS and antibiotics in 96-well plates and were left to equilibrate for 2 h. Cells were centrifuged within the microtiter plate (480 g, 4° C, 10 min) before medium was changed to RPMI 1640 without serum. After a further incubation period of 30 min to allow the lung cells to adjust to pH and temperature, cell toxicants were dissolved in RPMI 1640 [hydrogen peroxide (H_2O_2), *tert*-butylhydroperoxide (t-BuOOH), sodium nitroprusside (SNP)], or dimethylsulfoxide (paraquat, nitrofurantoin, menadione) and were added in a volume of 1 μl [dimethylsulfoxide (DMSO)] or 10 μl RPMI 1640. Incubation was continued for 18 h. Solvent alone was used as a control incubation and DMSO at a concentration of $< 1\%$ showed no cytotoxicity. Cytotoxicity was determined by LDH release or the MTT reduction capacity of lung cells.

Hepatocyte culture. 3.3×10^4 or 8×10^4 rat or murine hepatocytes, respectively, were plated in 200 μl RPMI 1640 with 10% newborn calf serum (NCS) and antibiotics in 96-well or 24-well plates, respectively. The cells were allowed to adhere for 4 h before the medium was changed to RPMI 1640 without serum. A preincubation period of 30 min allowed the cells to adjust to pH and temperature. Afterwards, hepatocytes were incubated with different toxic agents (dissolved in RPMI 1640 or DMSO) for 18 h, followed by assessment of cytotoxicity by the MTT reduction assay.

Viability and Cytotoxicity Assays

Viability of freshly isolated lung cells and hepatocytes was assessed by trypan blue exclusion (Phillips, 1973) and the viability of cells in culture was determined by LDH release or by the MTT assay as detailed below.

LDH was determined (Bergmeyer, 1984) in culture supernatants (S), and in the remaining cells (C) after lysis with Triton X-100. The percentage of LDH release was calculated as $S/(S+C)$. Alternatively, the capacity to produce formazan from MTT was measured essentially according to Mosmann (1983). Briefly, at the end of the incubation period with the toxicants, murine or rat hepatocytes were incubated with 0.4 or 0.7 mg/ml MTT, respectively, for 30 min. After removal of medium, reduced MTT was measured spectrophotometrically in an ELISA-reader at 560/690 nm after lysis of cells (containing formazan crystals) with 200 μl (murine hepatocytes) or 100 μl (rat hepatocytes) i-propanol/formic acid:95/5. To determine the formazan formation by murine hepatocytes, 100 μl of the solution containing lysed murine hepatocytes (cultured in 24-well plates) were transferred to 96-well microtiter plates. Similarly, rat lung cells were incubated with 1 mg/ml MTT for 4 h and were lysed by addition of 30 μl HCl/SDS (3 m*M*/3% final concentration). The maximum formazan production capacity of lung cells and hepatocytes immediately after plating or after several hours in culture (controls) was defined as 100% viability equivalent to a basal toxicity of 0%.

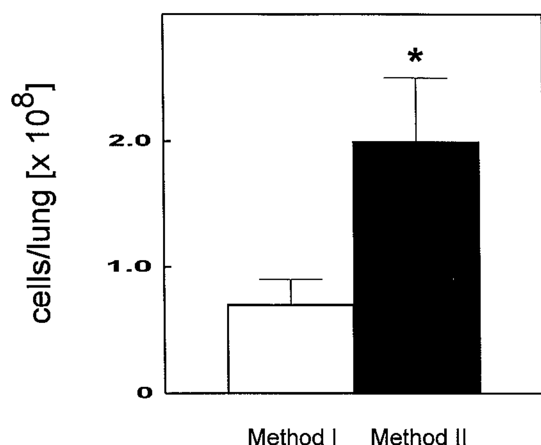

FIG. 1. Comparison of lung cell yields produced by instillation/immersion or recirculating perfusion of rat lungs. *Bars* show cell yields of lung cells after isolation by instillation/immersion (*open bar*; Method I) or by recirculating perfusion (*closed bar*; Method II). Viability of lung cells immediately after isolation was 70% (Method I) or 97% (Method II) as determined by trypan blue exclusion. Data represent means of 13 (Method I) or 17 (Method II) independent experiments ± SD. *Different from Method I by unpaired two-sided t-test, $P < 0.05$.

Cytochemistry

For cytochemical identification, cell smears were prepared on microscope slides and stained with May-Grünwald/Giemsa (Romeis, 1968), Papanicolaou (with and without acid alcohol) (Street, 1952), the myeloperoxidase method (Kaplow, 1965), or the periodic acid Schiff reaction (Hennemann, 1991). For determination of nonspecific esterase positive cells (Horwitz et al., 1977), staining of lung cells in suspension was followed by preparation of cell smears on microscope slides and microscopy.

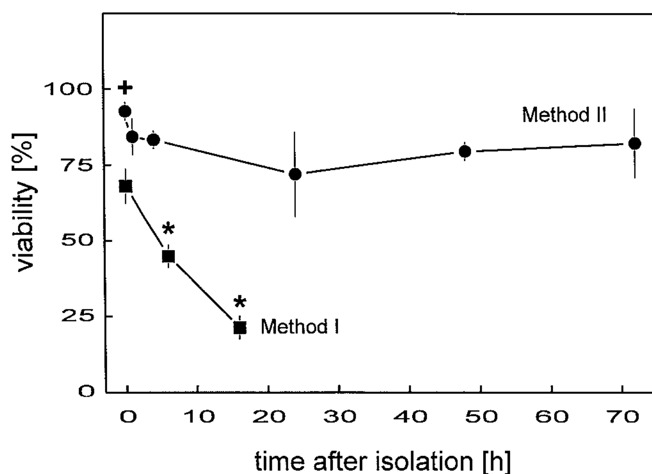

FIG. 2. Viability of lung cells various times after isolation. After isolation of lung cells by instillation and immersion (Method I) or by recirculating perfusion (Method II), 1.25×10^5 cells/well were plated in 250 µl medium onto 24-well plates (t = 0 h). Viability of lung cells was determined by LDH in supernatants at the times indicated. Medium was changed every 48 h. Data represent means of four (Method I) or three (Method II) independent experiments ± SD. *Different from previous time point by Repeated Measurement Anova, $P < 0.05$. +Different from Method I, $P < 0.05$.

Statistics

Data are expressed as means ± standard deviation (SD). All experiments were repeated at least three times. Normality and homoscedacity were analyzed using the Shapiro-Wilk test and the Bartlett test, respectively. Statistical significances were determined by an unpaired two-sided t-test if applicable, or with the unpaired Welch test (inhomogeneous variances). Time series were tested by Repeated Measurement Anova (GLM/REPEATED/PROFILE in SAS; Cary, NC). $P < 0.05$ was considered to be significant.

EC_{50} values were calculated from the concentration response curves using the program ALLFIT (De Lean et al., 1978). For comparison of the EC_{50} values of different toxic agents in the toxicity studies, the following groups were formed: H_2O_2 and t-BuOOH; paraquat, nitrofurantoin, and menadione; SNP. Concentration response curves of substances in one group were analyzed simultaneously, assuming that the maximum toxicity was 100% and the minimum toxicity was 0%. Identity of EC_{50} values and slopes was tested for by comparing the sum of squares from the constrained and the unconstrained model (F-test, $P < 0.05$). Therefore, if within one group different EC_{50} values are given (Table 1), they were significantly different from each other ($P < 0.05$).

RESULTS

Isolation and Culture of Rat Lung Cells

The first step of this study was to compare variants of a published procedure for isolating lung cells (Method I) with the method of enzymatic digestion by perfusion (Method II). Isolation by recirculating perfusion with different enzyme solutions (Method II) produced a cell yield of $2.0 \pm 0.5 \times 10^8$ cells per rat lung (i.e., more than twice as many cells as via isolation by instillation and immersion) (Fig. 1). Cells obtained by Method II were characterized by a superior viability (97%) compared to those obtained by Method I (70%; Fig. 2). The time course of viability of cultured lung cells was assessed by determination of LDH release (Fig. 2) or by trypan blue exclusion (data not shown, similar results). According to Method II and 72 h after isolation, the proportion of viable lung cells incubated in the presence of antibiotics and 10% FBS was > 75% compared to a viability of < 25% after 16 h for cells prepared according to Method I (Fig. 2). Beating of ciliated epithelial cells may be considered an alternative indicator of the quality of isolation and of the condition of lung cells in culture. Beating of ciliated cells isolated by Method II was observed for up to 5 d of cultivation, in contrast to cells isolated by instillation and immersion, where beating was noted only within the first 2 h after isolation. Two to four days after isolation, most of the lung cells were spread out and organized in a monolayer. Furthermore, from Day 4 in culture, proliferation of fibroblastlike cells was observed. According to these findings, it seemed appropriate to perform all further studies with cells isolated by Method II.

Heterogeneity of Rat Lung Cells Isolated by Recirculating Perfusion

Because the lung consists of many different cell types, heterogeneity becomes an important criterion for a representative lung cell culture. By light microscopy, many different cells with various cell sizes and cell shapes were seen suggesting a great variety of the isolated rat lung cells. In order to characterize some of these different cell types, cytochemical investigations were carried out. First, cells were stained with May-Grünwald/Giemsa and were identified by cell size, cell shape, nucleus shape, and nucleus/cytoplasm ratio. By this method, polymorphonuclear neutrophils, monocytes (with an irregular, indented nucleus), lymphocytes (with a large, circular nucleus),

and ciliated epithelial cells were identified. Furthermore, the large alveolar macrophages (cell diameter: ~ 15 μm determined by morphometry) with a circular nucleus and the smaller interstitial macrophages (cell diameter: ~ 8 μm) with an indented kidney-shaped nucleus were demonstrated. In contrast to alveolar macrophages that were esterase positive staining, interstitial macrophages stained negative or were only weakly esterase positive. Additionally, mucous and serous cells were demonstrated. Type II pneumocytes were identified by a modified Papanicolaou staining method. We then assessed the proportions of some of the identified cell populations in our lung cell culture. The percentage of alveolar macrophages was 4.9 ± 2.1% as determined by esterase staining. A percentage of 1.9% polymorphonuclear neutrophils as identified by myeloperoxidase staining was found in our cultures of isolated rat lung cells. By a modified Papanicolaou staining method a percentage of 19.5 ± 3.1% type II pneumocytes was detected. The classical Papanicolaou method failed to stain the type II cells in our lung culture system.

In addition, ciliated epithelial cells were identified as described above. Isolated pneumocytes were viable throughout an observation period of 5 d. In the first 2 d of cultivation, most of the cells began to adhere and to spread. However, from Day 4 on, a monolayer of various adherent lung cells and fibroblastlike cells was noted in our cultures.

Formazan Production Capacity of Lung Cells

Reduction of MTT has been shown to be a quick, simple, and sensitive method for assessment of viability (Mosmann, 1983). First, conditions for this test in our rat lung cell culture were optimized (*see* "Materials and Methods"). A linear dependency between the formazan production capacity and the cell number was obtained in experiments where lung cells were incubated with 1 mg/ml MTT for various times (Fig. 3). In order to explore the correlation between the MTT reduction assay and LDH release, lung cells were incubated 18 h with various concentrations of hydrogen peroxide (H_2O_2) and toxicity was determined by both LDH release and MTT assay. A linear correlation of the two parameters (Pearson correlation coefficient: > 0.91, $P < 0.001$; Fig. 3, *Inset*) was obtained. Similar comparative studies were performed with rat and mouse hepatocytes [correlation coefficients: > 0.95 (this study) and > 0.97 (Leist, 1993), respectively]. Under the conditions studied, H_2O_2 did not affect the reduction of MTT. The difference in basal cytotoxicity, when measured by LDH release compared to formazan formation, was due to different definitions of the time point zero (t = 0 h). Whereas in the case of LDH enzyme release into the medium before and during plating accounted for the basal toxicity, in the case of MTT only viable cells after plating contributed to reduction of the dye.

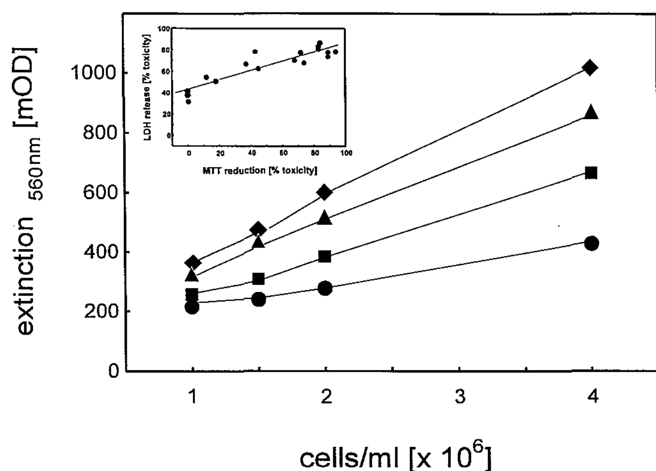

FIG. 3. Linearity of formazan reduction by isolated rat lung cells. After isolation and plating of cells in 200 μl medium onto 96-well plates, they were incubated for ● 0.5 h, ■ 1 h, ▲ 2 h and ◆ 4 h with 1 mg/ml MTT. After lysis, absorbance at 560/690 nm was determined. Data represent means of three independent experiments. All coefficients of variance were < 8.6%. The regression coefficient was > 0.98 for all curves shown. Inset: Correlation of MTT reduction and LDH release. After isolation, 4×10^5 cells/well were plated in 200 μl medium onto 96-well plates. 18 h after addition of H_2O_2 toxicity was assessed by measurement of LDH release and MTT reduction. Data represent means of triplicate samples from one experiment. The Pearson correlation coefficient was 0.91 with $P < 0.001$.

Toxicity Studies in Lung Cell and Hepatocyte Cultures

In order to evaluate the practical application of this lung cell system for *in vitro* testing, primary lung cell cultures were compared to conventional hepatocyte cultures from rats and mice with respect to the toxicity of various agents. For standardization, a specific lung cell isolation was accepted for toxicity studies when a viability of > 90% was reached and beating of cilia of ciliated epithelial cells was observed. Cells were incubated with the toxicants for 18 h and cytotoxicity was assessed by formazan production.

Fig. 4 shows that lung cells were about 10 times more susceptible to oxidative damage by H_2O_2 than hepatocytes from both rats and mice. The EC_{50} values for the oxidant H_2O_2 in different cell cultures were 0.61 m*M* for rat lung cells and 5.8 m*M* for rat and murine hepatocytes (Table 1). In contrast to H_2O_2-induced cytotoxicity, no significant differences in the toxicity of *tert*-butylhydroperoxide (t-BuOOH) on pulmonary compared to hepatic cells were observed (Fig. 4 and Table 1). The EC_{50} value for t-BuOOH in any cell culture was 100 μ*M*. All cell types were more sensitive to t-BuOOH than to H_2O_2 (Table 1).

Paraquat, a well-known lung toxicant *in vivo* (Clark et al., 1966), showed a biphasic toxic effect in lung cell cultures (Fig. 5). Whereas about 70% of the lung cells were noted to be very sensitive towards paraquat with an EC_{50} of 0.03 m*M*, the remaining 30% were much less susceptible towards this lung toxicant (EC_{50} > 10 m*M*). In murine or rat hepatocytes paraquat-induced cytotoxicity showed a conventional concentration dependency with larger EC_{50} values compared to the more sensitive lung cell population. Paraquat was more toxic for murine hepatocytes (EC_{50} = 0.18 m*M*) than for rat hepatocytes (EC_{50} = 2.76 m*M*; Fig. 5 and Table 1).

The results obtained with the redox cyclers menadione and nitrofurantoin are given in Table 1. Menadione was the most cytotoxic compound tested in our studies with EC_{50} values in the micromolar range in lung cells and rat hepatocytes. A similar profile of cytotoxicity was obtained with nitrofurantoin with an EC_{50} of an order of magnitude greater than the one of menadione. Murine hepatocytes were less sensitive towards either redox cycler (Table 1).

Sodium nitroprusside (SNP) was not toxic for lung cells up to concentrations of 1 m*M* (Fig. 6). Toxicity of SNP showed a steep concentration response curve between 1 m*M* and 20 m*M* (EC_{50} = 2.62 m*M*; Table 1). Rat or murine hepatocytes were 15 or 87 times, respectively, more susceptible to this NO donor than lung cells. The

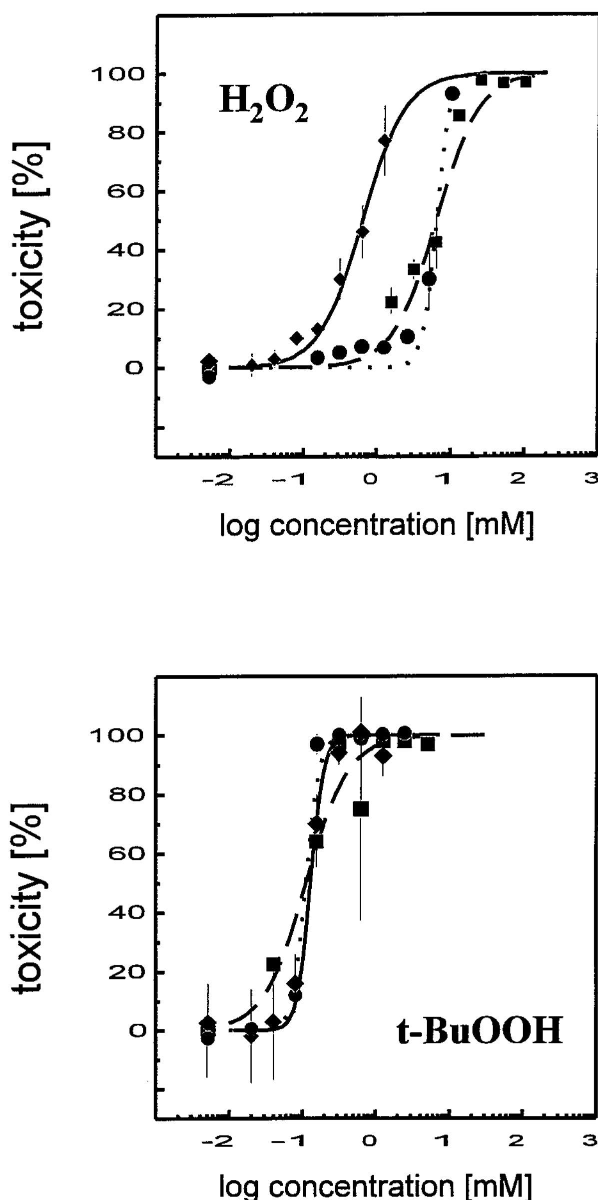

FIG. 4. Concentration dependency of hydrogen peroxide (H_2O_2) and *tert*-butylhydroperoxide (t-BuOOH) toxicity in rat lung cell cultures and hepatocyte cultures from rats and mice. After isolation of rat lung cells and hepatocytes from rats and mice, cells were incubated with various concentrations of H_2O_2 or t-BuOOH. The toxicity at 18 h was assessed by the MTT assay. Data represent means of triplicate samples from one representative experiment ± SD. ◆ (*solid line*): rat lung cells; ■ (*dashed line*): rat hepatocytes; ● (*dotted line*): murine hepatocytes.

EC_{50} values were 0.03 m*M* or 0.17 m*M* for murine or rat hepatocytes, respectively. These observations suggest that hepatocytes are essentially more sensitive to NO-releasing compounds than lung cells.

DISCUSSION

Primary cell cultures are considered to be useful models for studying mechanistic aspects of pharmacology and toxicology at a cellular level. In contrast to major advances in the understanding of hepatic processes derived from studies with hepatocyte cultures, isolated pneumocytes have received little attention, with the notable exception of alveolar macrophages and type II pneumocytes. The isolation of mixed lung cells from rat lungs has only rarely been described (Devereux and Fouts, 1989; Dobbs, 1990; Lavnikova et al., 1993). These procedures of isolation were based on instillation, immersion, and digestion of dissected lungs. In our hands, this method for isolating rat lung cells (Method I), resulted in low cell yields with moderate cell viability. Variations of enzyme composition, activity, or incubation times failed to increase either cell yield or viability in our experiments (data not shown). Therefore, we adapted and optimized the standard technique for isolating hepatocytes (i.e., perfusion with proteolytic enzymes) (Seglen, 1976) for the isolation of pneumocytes (Method II).

Isolation of lung cells by perfusion with digestion enzymes significantly increased cell yield and viability. The improvements made with Method II may be explained by the exposure of a greater surface area to proteolytic enzyme activity when perfused through the pulmonary artery. The enzyme concentrations were well tolerated by lung cells as judged by the high viability and the continued beating of cilia of epithelial brush border cells. Thus, this isolation method might also be suitable for studies where high numbers of cells are required, such as for elutriation of certain cell populations.

One of the reasons that mixed isolated pneumocytes have received little attention in the past is that lungs contain more than 40 different cell types (Sorokin, 1970). An optimal isolation procedure should guarantee survival of all cells. Therefore, in addition to cell yield and viability, heterogeneity of isolated lung cells is an important criterion. This cellular heterogeneity of the lung cell culture appeared to be largely conserved by our method as demonstrated by staining with May-Grünwald/Giemsa and by specific stains for different major lung cell types. There were cell types that could not be discriminated with the available staining techniques. The proportions of the identified lung cells, ~20% type II pneumocytes, ~5% alveolar macrophages, and ~2% polymorphonuclear neutrophils in our system, compare well to previous observations of about 12–14% type II cells (Crapo et al., 1980; Pinkerton et al., 1982), 3–4% alveolar macrophages (Crapo et al., 1980; Pinkerton et al., 1982), and

TABLE 1

EC_{50} VALUES OF VARIOUS TOXIC AGENTS IN LUNG CELL CULTURES FROM RATS AND HEPATOCYTE CULTURES FROM RATS AND MICE

Toxicant[a]	EC_{50} [m*M*] Rat Lung Cells	EC_{50} [m*M*] Rat Hepatocytes	EC_{50} [m*M*] Mouse Hepatocytes
H_2O_2	0.61	5.80	5.80
t-BuOOH	0.11	0.11	0.11
Menadione	0.006	0.006	0.04
Nitrofurantoin	0.04	0.04	0.18
Paraquat[b]	0.03/>10	2.76	0.18
SNP	2.62	0.17	0.03

[a]For comparison of the EC_{50} values of different toxic agents, the following groups were formed: H_2O_2 and t-BuOOH; paraquat, menadione, and nitrofurantoin; SNP.

[b]Due to the biphasic toxic effect in the lung cell culture, two EC_{50} values for paraquat are given.

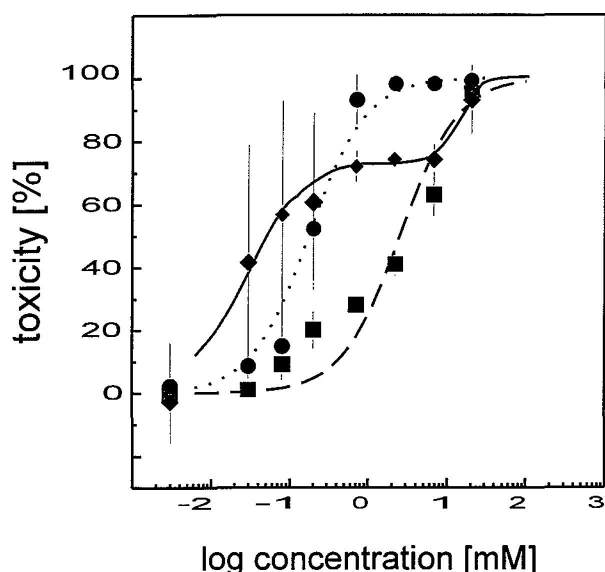

FIG. 5. Concentration dependency of paraquat toxicity in rat lung cell cultures and hepatocyte cultures from rats and mice. After isolation of rat lung cells and hepatocytes from rats and mice, cells were incubated with various concentrations of paraquat. The toxicity at 18 h was assessed by the MTT assay. Data represent means of triplicate samples from one representative experiment ± SD. ◆ (*solid line*): rat lung cells; ■ (*dashed line*): rat hepatocytes; ● (*dotted line*): murine hepatocytes.

~8% polymorphonuclear neutrophils (Lehnert et al., 1985) in rat lungs.

Furthermore, granula (lamellar bodies) of type II cells were noted up to Day 4 in culture. In agreement with published data (Evans et al., 1973; Adamson and Bowden, 1974; Kikkawa and Yoneda, 1974; Diglio and Kikkawa, 1977; Paine et al., 1990), no granula were detected on Day 5. Several groups have reported that type II cells cultured for 5 d differentiate to type I pneumocytes [medium with serum (Paine et al., 1990)] accompanied by loss of lamellar bodies (rat: Evans et al., 1973; Kikkawa and Yoneda, 1974; Adamson and Bowden, 1975; rabbit: Diglio and Kikkawa, 1977; mouse: Adamson and Bowden, 1974). These results indicate that proliferation and changes in morphology of cultured rat lung cells isolated by recirculating perfusion are similar to those of cells isolated by other methods.

When we adopted the method of MTT reduction for toxicity assessment in our lung cell system we noted, during microscopic inspection, that the dye reduction capacity of different lung cell types was different in intensity and duration. Because the contribution of MTT reduction capacity of single lung cell types could not be determined, the MTT signal has to be taken as an average measurement of pulmonary cytotoxicity. The steep and uniform concentration response curves obtained for most toxicants suggest that the different lung cell types react similarly to the exposure to these agents, with the notable exception of paraquat.

Menadione, paraquat, nitrofurantoin, t-BuOOH, and H_2O_2 were toxic for rat lung cells in concentrations < 0.1 m*M*. Although H_2O_2 was the least toxic oxidant for lung cells, it was 10 times more toxic for isolated pneumocytes than for isolated rat and murine hepatocytes. This indicates that lung cells have less antioxidant activity

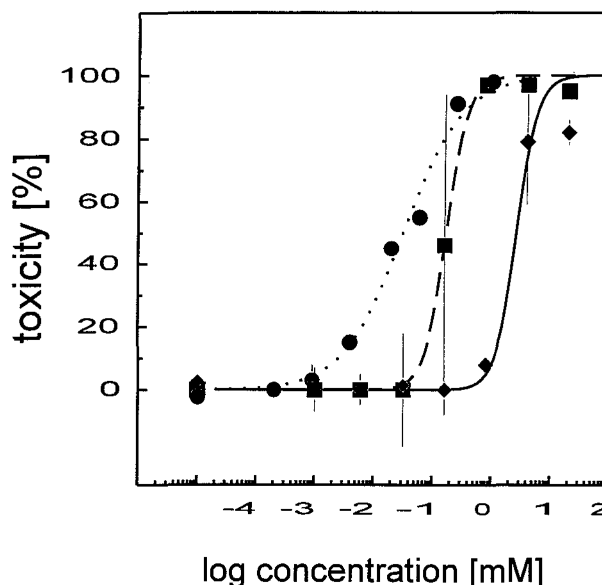

FIG. 6. Concentration dependency of sodium nitroprusside (SNP) toxicity in rat lung cell cultures and hepatocyte cultures from rats and mice. After isolation of rat lung cells and hepatocytes from rats and mice, cells were incubated with various concentrations of SNP. The toxicity at 18 h was assessed by the MTT assay. Data represent means of triplicate samples from one representative experiment ± SD. ◆ (*solid line*): rat lung cells; ■ (*dashed line*): rat hepatocytes; ● (*dotted line*): murine hepatocytes.

than hepatocytes. Indeed, low catalase values in rat lungs compared to lungs from man, baboon, or hamster have been reported (Bryan and Jenkinson, 1987). Toxicity of t-BuOOH was similar for hepatocytes and pneumocytes. Both cell types were more sensitive to t-BuOOH than to H_2O_2, which suggests similar mechanisms of t-BuOOH toxicity in lung and liver cells. The large catalase/glutathione peroxidase ratio in hepatocytes (Ito et al., 1992) and lung cells (Bryan and Jenkinson, 1987) might be responsible for the relatively low toxicity of H_2O_2 compared to t-BuOOH.

From previous studies with nitrofurantoin, it was concluded that the *in vivo* toxicity of this compound in rat lungs impairs detoxification of reactive oxygen species by decreasing catalase and glutathione reductase activity and hence inducing oxidative stress and lipid peroxidation (Suntres and Shek, 1992). Similar results were obtained in freshly isolated rat hepatocyte cultures, where nitrofurantoin caused oxygen activation as a result of redox cycling followed by complete GSH depletion and cell death (Silva et al., 1991, 1993). In hepatocytes, menadione was shown to react with GSH forming an adduct that was released into the medium (Di Monte et al., 1984) and can still undergo redox cycling (Takahashi et al., 1987) (i.e., form superoxide and induce lipid peroxidation, enzyme inactivation, GSH depletion, and DNA damage) (Kappus, 1986). It seems possible that mechanisms similar to those described for hepatocytes are responsible for the toxicity of menadione in pneumocytes. Our observations that the toxicity of menadione as well as of nitrofurantoin was clearly higher in rat cells compared to murine cells may be related to low catalase concentrations in rat organs compared to organs from other species (Bryan and Jenkinson, 1987).

In contrast to the higher susceptibility of lung cells generally observed in our study, we also noted one radical generating toxicant that was more toxic towards hepatocytes than towards pneumocytes (i.e., SNP). The NO donor SNP is known to induce apoptotic processes in macrophages (Meβmer and Brüne, 1994) and ADP ribosylation in pancreatic cells (Dimmeler et al., 1993). In agreement with a recent study showing that hepatocytes were more sensitive to SNP toxicity than various other cell lines (Kröncke et al., 1993), we also observed that hepatocytes were more sensitive to this compound than primary lung cells. Furthermore, EC_{50} values of SNP in rat and murine hepatocyte cultures obtained in this study agreed with data from literature [rat: $EC_{50} = 300\ \mu M$ (Kröncke et al., 1993); mouse: $EC_{50} = 32\ \mu M$ (Leist, 1993)].

The sensitivity of lungs to paraquat *in vivo* is primarily due to the pharmacokinetic properties of this compound. Paraquat is actively taken up by alveolar cells by a diamine/polyamine transport system where it undergoes NADPH-dependent one-electron reduction to form free radicals capable of reacting with molecular oxygen (Smith, 1987). The uptake of high concentrations of paraquat has also been demonstrated in lung tissue slices (Rose et al., 1976). In our study, we observed that 70% of the isolated pneumocytes were very sensitive to paraquat, whereas 30% were only as sensitive as hepatocytes from rats and mice (Fig. 5). These results demonstrate that the organ specific toxicity of paraquat is conserved in primary pneumocyte culture and that a selectivity of this toxicity for a certain cell population exists. As yet, the sensitive pneumocyte population(s) remain(s) to be identified.

In summary, the high viability of the isolated lung cells, together with their heterogeneity, differential, and characteristic sensitivity to toxicants and the fact that they could be maintained in culture for several days, would suggest that the culture system described here is suitable for studying lung toxicology and pharmacology.

References

Adamson, I. Y. R.; Bowden, D. H. The type II cell as a progenitor of alveolar epithelial regeneration. A cytodynamic study in mice after exposure to oxygen. Lab. Invest. 30:35–42; 1974.

Adamson, I. Y. R.; Bowden, D. H. Derivation of type I epithelium from type II cells in the developing rat lung. Lab. Invest. 32:736–745; 1975.

Batenburg, J.; Otto-Verberne, C.; Have-Opbroek, A., et al. Isolation of alveolar type II cells from fetal rat lung by differential adherence in monolayer culture. Biochim. Biophys. Acta 960:441–453; 1988.

Bergmeyer, H. U. Methods of enzymatic analysis. Weinheim, Germany: Verlag Chemie; 1984.

Berk, J. L.; Franzblau, C.; Goldstein, R. H. Recombinant interleukin-1β inhibits elastin formation by a neonatal rat lung fibroblast subtype. J. Biol. Chem. 266:3192–3197; 1991.

Brown, L.; Longmore, W. Altered phospholipid secretion in type II pneumocytes isolated from streptozotocin-diabetic rats. Biochim. Biophys. Acta 878:258–265; 1986.

Bryan, C. L.; Jenkinson, S. G. Species variations in lung antioxidant enzyme activities. J. Appl. Physiol. 63:597–602; 1987.

Clark, D. G.; McElligot, T. F.; Hurst, E. W. The toxicity of paraquat. Br. J. Ind. Med. 23:126–132; 1966.

Crapo, J. D.; Barry, B. E.; Foscue, H. A., et al. Structural and biochemical changes in rat lungs occurring during exposures to lethal and adaptive doses of oxygen. Am. Rev. Respir. Dis. 122:123–145; 1980.

De Lean, A.; Munson, P. J.; Rodbard, D. Simultaneous analysis of families of sigmoidal curves: Application to bioassay, radioligand assay, and physiological dose-response curves. Am. J. Physiol. 235:E97–E102; 1978.

Devereux, T. R.; Fouts, J. R. Isolation of pulmonary cells and use in studies of xenobiotic metabolism. Methods Enzymol. 77:147–154; 1989.

Diglio, C. A.; Kikkawa, Y. The type II epithelial cells of the lung. IV. Adaptation and behavior of isolated type II cells in culture. Lab. Invest. 37:622–631; 1977.

Dimmeler, S.; Ankarcrona, M.; Nicotera, P., et al. Exogenous nitric oxide (NO) generation or IL-1 β-induced intracellular NO production stimulates inhibitory auto-ADP-ribosylation of glyceraldehyde-3-phosphate dehydrogenase in RINm5F cells. J. Immunol. 150:2964–2971; 1993.

Di Monte, D.; Ross, D.; Bellomo, G., et al. Alterations in intracellular thiol homeostasis during the metabolism of menadione by isolated rat hepatocytes. Arch. Biochem. Biophys. 235:334–342; 1984.

Dobbs, L. G. Isolation and culture of alveolar type II cells. Am. J. Physiol. 258:L134–L147; 1990.

Dobbs, L.; Mason, R. Pulmonary alveolar type II cells isolated from rats. Release of phosphatidylcholine in response to β-adrenergic stimulation. J. Clin. Invest. 62:378–387; 1979.

Dobbs, L. G.; Gonzalez, R.; Williams, M. C. An improved method for isolating type II cells in high yield and purity. Am. Rev. Respir. Dis. 134:141–145; 1986.

Douglas, W. H.; Kaighn, M. E. Clonal isolation of differentiated rat lung cells. In Vitro 10:230–237; 1974.

Douglas, W. H. J.; Teel, R. W. An organotypic *in vitro* model system for studying pulmonary surfactant production by type II alveolar pneumocytes. Am. Rev. Respir. Dis. 113:17–23; 1976.

Evans, M. J.; Cabral, L. J.; Stephens, R. J., et al. Renewal of alveolar epithelium in the rat following exposure to NO_2. Am. J. Pathol. 70:175–198; 1973.

Finkelstein, J.; Maniscalco, W.; Shapiro, D. Properties of freshly isolated type II alveolar epithelial cells. Biochim. Biophys. Acta 762:398–404; 1983.

Goodman, B.; Fleischer, R.; Crandall, E. Evidence for active Na^+ transport by cultured monolayers of pulmonary alveolar epithelial cells. Am. J. Physiol. 245:C78–C83; 1983.

Gould, M. R.; Clements, J. A.; Jones, A. L., et al. Dispersal of rabbit lung into individual viable cells: A new model for the study of lung metabolism. Science 178:1209–1210; 1972.

Hartung, T.; Wendel, A. Endotoxin-inducible cytotoxicity in liver cell cultures I. Biochem. Pharmacol. 42:1129–1135; 1991.

Hennemann, H. H. Periodic acid schiff reaction. In Hämatologie-Lehrbuch und Atlas. Stuttgart, Germany: Schattauer Verlag; 1991:166–167.

Hinz, R. W.; Syverton, J. T. Mammalian cell cultures for study influenza virus. I. Preparation of monolayer cultures with collagenase. Proc. Soc. Exp. Biol. Med. 101:19–31; 1959.

Horwitz, D. A.; Allison, A. C.; Ward, P., et al. Identification of human mononuclear leukocyte populations by esterase staining. Clin. Exp. Immunol. 30:289–298; 1977.

Ito, Y.; Hiraishi, H.; Razandi, M., et al. Role of cellular superoxide dismutase against reactive oxygen metabolite-induced cell damage in cultured hepatocytes. Hepatology 16:247–254; 1992.

Jassal, D.; Han, R. N. N.; Caniggia, I., et al. Growth of distal fetal rat lung epithelial cells in a defined serum-free medium. In Vitro Cell. Dev. Biol. 27A:625–632; 1991.

Kaplow, L. S. Simplified myeloperoxidase stain using benzidine dihydrochloride. Blood 26:215–219; 1965.

Kappus, H. Overview of enzyme systems involved in bio-reduction of drugs and in redox cycling. Biochem. Pharmacol. 35:1–6; 1986.

Kikkawa, Y.; Yoneda, K. Type II epithelial cell of the lung. I. Method of isolation. Lab. Invest. 30:76–84; 1974.

Klaunig, J. E.; Goldblatt, P. J.; Hinton, D. E., et al. Mouse liver cell culture I. Hepatocyte isolation. In Vitro 17:913–925; 1981a.

Klaunig, J. E.; Goldblatt, P. J.; Hinton, D. E., et al. Mouse liver cell culture II. Primary culture. In Vitro 17:926–934; 1981b.

Kröncke, K. D.; Brenner, H. H.; Rodriguez, M. L., et al. Pancreatic islet cells are highly susceptible towards the cytotoxic effects of chemically generated nitric oxide. Biochim. Biophys. Acta 1182:221–229; 1993.

Lavnikova, N.; Prokhorova, S.; Helyar, L., et al. Isolation and partial characterization of subpopulations of alveolar macrophages, granulocytes, and highly enriched interstitial macrophages from rat lung. Am. J. Respir. Cell Mol. Biol. 8:384–392; 1993.

Lehnert, B. E.; Valdez, Y. E.; Holland, L. M. Pulmonary macrophages: Alveolar and interstitial populations. Exp. Lung Res. 9:177–190; 1985.

Leist, M. Inflammatory stimuli and mediators in the mouse liver. Konstanz, Germany Hartung-Gorre Verlag; 1993.

Ma, J. Y. C.; La Cagnin, L. B.; Bowman, L., et al. Carbon tetrachloride inhibits synthesis of pulmonary surfactant disaturated phosphatidylcholines and ATP production in alveolar type II cells. Biochim. Biophys. Acta 1003:136–144; 1989.

Mangum, J.; Everitt, J.; Bonner, J., et al. Co-culture of primary pulmonary cells to model alveolar injury and translocation of proteins. In Vitro 26:1135–1143; 1990.

Mason, R.; Williams, M.; Greenleaf, R., et al. Isolation and properties of type II alveolar cells from rat lung. Am. Rev. Respir. Dis. 115:1015–1026; 1977.

Meβmer, U. K.; Brüne, B. Apoptosis and DNA fragmentation in RAW 264.7-macrophages induced by nitric oxide releasing compounds. Naunyn-Schmiedeberg's Arch. Pharmacol. 349 (Supplement): R 26, 1994.

Mosmann, T. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assay. J. Immunol. Methods 65:55–56; 1983.

Paine, R.; Joyce-Brady, M.; Clement, A., et al. Serum accelerates the loss of type II cell differentiation *in vitro*. Am. J. Respir. Cell Mol. Biol. 3:311–323; 1990.

Phillips, H. J. Dissociation of single cells from lung or kidney tissue with elastase. In Vitro 8:101–105; 1972.

Phillips, H. J. Dye exclusion tests for viability. In: Kruse, P. F., Jr.; Patterson, M. K., Jr., eds. Tissue culture methods and applications. New York: Academic Press; 1973:406–408.

Pinkerton, K. E.; Barry, B. E.; O'Neil, J. J., et al. Morphologic lung changes in the lifespan of Fisher 344 rats. Am. J. Anat. 164:155–174; 1982.

Post, M.; Torday, J. S.; Smith, B. T. Alveolar type II cells isolated from fetal rat lung organotypic cultures synthesize and secrete surfactant-associated phospholipids and respond to fibroblast-pneumocyte factor. Exp. Lung Res. 7:53–65; 1984.

Romeis, B. Panoptische Färbung nach Pappenheim. In Mikroskopische Techniken. München, Germany: Verlag Leibniz; 1968:475.

Rose, M. S.; Lock, E. A.; Smith, L. L., et al. Paraquat accumulation: Tissue and species specificity. Biochem. Pharmacol. 25:419–423; 1976.

Seglen, P. O. Preparation of isolated rat liver cells. Methods Cell Biol. 13:29–83; 1976.

Sikpi, M. D.; Nair, C. R.; Johns, A. E., et al. Metabolic and ultrastructural characterization of guinea-pig alveolar type II cells isolated by centrifugal elutriation. Biochim. Biophys. Acta 877:20–30; 1986.

Silva, J. M.; Khan, S.; O'Brien, P. J. Molecular mechanisms of nitrofurantoin-induced hepatocyte toxicity in aerobic versus hypoxic conditions. Arch. Biochem. Biophys. 305:362–369; 1993.

Silva, J. M.; McGirr, L.; O'Brien, P. J. Prevention of nitrofurantoin-induced cytotoxicity in isolated hepatocytes by fructose. Arch. Biochem. Biophys. 289:313–318; 1991.

Smith, L. L. The mechanism of paraquat toxicity in the lung. Biochem. Toxicol. 8:37–71; 1987.

Sorokin, S. P. The cells of the lung. In: Nittesheim, P.; Hanna, M. G., Jr.; Deatherage, J. W., Jr., eds. Conference on the morphology of experimental respiratory carcinogenesis. Washington, DC: US Atomic Energy Commission; 1970:3–41.

Street, C. M. Papanicolaou technique in exfoliative cytology. In: Cowdry, E. V., ed. Laboratory techniques in biology and medicine. Baltimore: Williams & Wilkins; 1952:253–259.

Suntres, Z. E.; Shek, P. N. Nitrofurantoin-induced pulmonary toxicity. *In vivo* evidence for oxidative stress-mediated mechanisms. Biochem. Pharmacol. 43:1127–1135; 1992.

Takahashi, N.; Schreiber, J.; Fischer, V., et al. Formation of glutathione conjugated semiquinones by the reaction of quinones with glutathione: An ESR study. Arch. Biochem. Biophys. 252:41–48; 1987.

Van Overveld, F. J.; Brujnzeel, P. L. B.; Kreukniet, J., et al. The isolation of inflammatory cells from human lung tissue. Int. J. Tiss. React. IX:61–67; 1987.