Clara Cell Cultures from the Mouse and Their Reaction to Bronchiolar Toxins

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The major aim of this study was to determine if small numbers of freshly isolated mouse Clara cells could be used to rapidly screen the toxic effects of a number of diverse pulmonary toxins. A short-term (20 hr) culture of functionally competent (nitotetrazolium reductase positive) Clara cells was developed. In this culture the Clara cells were allowed to attach to an extracellular matrix in 96-well multiwell plates containing a culture medium of DCCM 1 and Ultroser G (0.4%). Pulmonary toxins (a total of 26 agents with concentrations ranging from $10^{-7}$ M to $10^{-3}$ M) were examined for their ability to reduce the attachment efficiency of functionally competent Clara cells and TD$_{50}$ values (the amount of toxin required to reduce normal attachment efficiency by 50%) were calculated. With the possible exception of some halogenated hydrocarbons, the simple toxicity test in vitro correlated well with the known effects of the bronchiolar necrotic agents in vivo. For 13 compounds studied there was a direct correlation between TD$_{50}$ values in vitro and LD$_{50}$ values (mostly oral) in rodents in vivo, the correlation coefficient of the regression line being 0.783.

Introduction

A number of low molecular weight chemicals (50–300 Da) have been shown to damage the lung (Table 1). In the last decade it has been recognized that more and more of these chemically diverse compounds initiate necrosis in the Clara cell population of the lung. Indeed, for some compounds the Clara cell is the most sensitive target, perhaps because it has a high complement of P-450 (and other phase 1) enzymes that initiate the metabolism of lipophilic and some hydrophilic substances. Herein lies a problem for the Clara cell. While it has the potential to catabolize parent compounds to more soluble (and hence more easily removed) metabolites, some of these intermediates may destroy the cell.

To date, most Clara cell/toxin interaction studies have been carried out in vivo (Table 1), possibly because there is some concern that in vitro systems are inadequate; in toxicity studies in vitro systems can provide false positive/negative results. Nevertheless, a useful in vitro screening system, apart from reducing the number of experimental animals, offers a number of advantages. These would include a rapid, inexpensive test, an opportunity to test multiple concentrations, or the ability to rapidly screen combinations of chemicals or inhibitory agents. In this last aspect, a useful in vitro system provides ideal conditions for mechanistic studies (24).

The major aim of this study was to determine if small numbers of freshly isolated mouse Clara cells (24) could be used to rapidly screen the toxic effects of a number of chemically diverse pulmonary toxins (Table 1). Prior to carrying out the toxicity study, a number of experiments were carried out to determine the best manner in which to keep the Clara cells in short-term culture.

Few studies have been carried out on the maintenance of Clara cells in culture, although an early brief report by Patton et al. (25) indicated that collagen I or IV assisted cell attachment when cultures were maintained in Ham's F12 medium. Other investigators (26) have shown that Clara cells preferentially attach to a collagen matrix support that has been preconditioned by tracheal cells. The importance of extracellular matrices for the culture of certain cell types has been recently reviewed (27). In addition, the use of chemically defined media (supplemented with growth factors and hormones) (28,29) or serum substitute medium (30) for maintaining epithelial cells has gained increasing attention in recent years. Thus, in the present study, the effect of different substrata (plastic, fibronectin, collagens I and IV, extracellular matrix) and a variety of culture media (Ham's F12 with and without hormone and growth factor supplements, low and high concentrations of fetal bovine...
Table 1. Characteristics (I) of chemicals that damage the lung and LD₅₀ values* a in rodents.

| Chemical             | Molecular weight | Soluble in water | Soluble in methanol | P-50% activation | Glutathione conjugation | Clara cell necrosis | Other lung cell damage | Reference | LD₅₀ in vivo (mg/kg) | Route of administration | Rodent |
|----------------------|------------------|------------------|---------------------|------------------|------------------------|---------------------|-----------------------|-----------|----------------------|-------------------------|--------|
| Bromobenzene         | 157              | +                | +                   | +                | +                      | (3)                 |                       |           | 1000                  | IP          | M            |
| Chlorobenzene        | 112              | +                | +                   | +                | +                      | (4)                 |                       |           | 2910                  | o           | R            |
| 1-2 Dichlorobenzene  | 147              | +                | +                   | +                | +                      | (4)                 |                       |           | 500                   | o           | R            |
| 1-3 Dichlorobenzene  | 147              | +                | +                   | +                | +                      | (4)                 |                       |           | 2950                  | o           | M            |
| 1-4 Dichlorobenzene  | 147              | +                | +                   | +                | +                      | (5)                 |                       |           |                      |             |              |
| Dichloromethylene    | 97               | +                | +                   | +                | +                      | (6)                 | 294                   |           | 2402                  | o           | M            |
| Trichloroethylene    | 131              | +                | +                   | +                | +                      | (7)                 | 294                   |           | 589                   | o           | M            |
| Naphtalene           | 128              | +                | +                   | +                | +                      | (8,9)               | 294                   |           | 38                    | o           | M            |
| 4-Ipomeanol          | 155              | +                | +                   | +                | +                      | (9,10,11)           | 294                   |           | 175                   | ip          | M            |
| 3-Methylindole       | 131              | +                | +                   | +                | +                      | (12,13)             | 294                   |           | 960                   | o           | R            |
| Nitrosodiethylamine  | 102              | +                | +                   | +                | +                      | (14,15)             | 294                   |           | 2500                  | o           | M            |
| Urethane             | 89               | +                | +                   | +                | +                      | (15)                |                       |           |                       |             |              |
| Methylcholanthrene   | 268 d            | +                | +                   | +                | +                      | (16)                | 294                   |           | 1040                  | o           | M            |
| Butylated hydroxytoluene | 220  c       | +                | +                   | +                | +                      | 294                 |                       |           | 137                   | o           | M            |
| Cyclophosphamide     | 279              | +                | +                   | +                | +                      | (17)                | 294                   |           | 40                    | o           | M            |
| Acrolein             | 56               | +                | +                   | +                | +                      | (18)                | 294                   |           |                       |             |              |
| Bulsulfan            | 246              | +                | +                   | +                | +                      | (19)                | 294                   |           | 233                   | o           | M            |
| Alloxan              | 169              | +                | +                   | +                | +                      | (20)                | 294                   |           | 60                    | o           | M            |
| Paraquat             | 257              | +                | +                   | +                | +                      | (21)                | 294                   |           |                       |             |              |
| Diquat               | 362              | +                | +                   | +                | +                      | (22)                | 294                   |           |                       |             |              |
| Spermidine           | 254              | +                | +                   | +                | +                      | (23)                | 294                   |           |                       |             |              |
| Cadmium chloride     | 228              | +                | +                   | +                | +                      | (24)                | 294                   |           | 60                    | o           | M            |
| Potassium dichromate  | 294              | +                | +                   | +                | +                      | (25)                | 294                   |           |                       |             |              |
| Nickel chloride      | 238              | +                | +                   | +                | +                      | (26)                | 294                   |           |                       |             |              |
| Zinc chloride        | 136              | +                | +                   | +                | +                      | (27)                | 294                   |           |                       |             |              |
| Ammonium sulfate     | 132              | +                | +                   | +                | +                      | (21–29)             | 294                   |           |                       |             |              |

*LD₅₀ values given for oral (o) or intraperitoneal (IP) dosing in mouse (M) or rat (R) (2).

**Solubilized in dimethylformamide (final concentration 0.5%).

Solubilized in ethanol (final concentration 0.5%).

Inhalation (personal communication Dr. I. Wyatt, ICI PLC, Cheshire, UK).

serum, single strength and concentrated serum substitutes) on the attachment efficiency of functionally competent [nitrotetrazolium positive (24)] Clara cells was determined. Once a combination of medium and substrate that gave a high efficiency of attachment had been achieved, the effect of 26 chemicals (Table 1) on this process was monitored.

Materials and Methods

Culture Media

Ham's F12 medium was obtained from Sigma (Poole, Dorset, UK) and was used direct following the addition of ascorbate (25 μg/mL), penicillin (60 μg/mL), and gentamicin (50 μg/mL). This was designated F12 medium. F12 was also supplemented with hormones and growth factor. The resultant medium, designated F12/H, contained F12 plus insulin (10 μg/mL), transferrin (10 μg/mL), epidermal growth factor (25 μg/mL), and 0.4% hypotonic extract. F12 and F12/H were mixed with varying amounts of fetal bovine serum (Flow Laboratories) or with Ultrosur G. Ultrosur G is a serum substitute medium containing growth factors and protein; it was obtained from IBF Biotechnics (Villeneuve-la-Gaverne, France) (now available from Gibco). DCCM 1 is a single-strength serum substitute, which contains protein but no growth factors. The exact composition of this medium, available through Biological Industries Ltd. (Cumbernauld, Glasgow, Scotland) has not been disclosed. DCCM 1 was mixed with 2% fetal bovine serum or with 0.4% Ultrosur in some of the experiments.

Preparation of Substrata in Multiwells

Ninety-six-well multiwell plates were purchased from Costar (Northumbria Biological Ltd., Cramlington, UK) and coated with different attachment materials. From stock solutions of bovine serum albumin (10 μg/mL, Sigma), fibronectin (10 μg/mL, Sigma), or Vitrogen (30 μg/mL, Flow Laboratories Rickmansworth, Herts., UK), 30 μL was added to each well and allowed to dry overnight at room temperature in an ultraviolet light cabinet.
For a Vitrogen/fibronectin coating, the collagen was added first, followed by the fibronectin. None of the coated plates were washed prior to use. Extracellular matrix (ECM) coated 96-well multiwell plates were purchased from Biological Industries Ltd. The ECM was derived from an endothelial cell line, and the plates were checked to ensure the absence of any cells and then washed before use with phosphate-buffered 0.15 M NaCl.

**Attachment Efficiency Measurement**

The purity of freshly prepared mouse Clara cell isolates was assessed from cytospin preparations using the nitrotetrazolium (NBT) staining technique, previously described (24). Invariably, 65 to 70% of the total cells were strongly NBT positive. Thus, the actual number of Clara cells in 50,000 total cells dispersed into each multiwell at time 0 could be calculated ( = Z). After incubation of the cells at 37°C in 95% air and 5% CO₂ for different time periods (approximately 20 hr and 40 hr), the medium was removed, the wells were washed to remove loosely attached cells, and those that were attached, stained with NBT (24). The cultures were examined using a reverse microscope, and the total number of NBT-positive cells in the field of view were recorded (= F). This procedure was repeated four or five times in other fields of view of the culture (to count 800–1000 cells) and a mean number of NBT-positive cells/field view obtained ( = F_m). The area covered by the field of view as a proportion of the area of the base of the well was determined and a factor K calculated for the number of fields of view (by area) present in each well. Attachment efficiency of the Clara cells in different media and substrata was calculated from the formula:

\[-F_m/Z \times K \times 100\%\]

**Preparations of Toxins and Toxicity Assay**

Stock solutions of each toxin in DCCM 1 medium were made up at 200 times the highest concentration required for the experiment (usually 2 \times 10^{-2} M). This was serially diluted (10-fold each step) to obtain two times the lowest concentration required (usually 2 \times 10^{-7} M). For lipophilic compounds (Table 1), the stock solution was made up in 100% methanol, and upon dilution with DCCM 1, the working concentration of methanol was 0.5% in each well. Freshly isolated Clara cells were equilibrated in plastic Petri dishes in DCCM 1 medium for 2 hr. Contaminating cells such as macrophages or fibroblasts attached to the plastic, but the Clara cells remained in suspension. The Clara cells were removed and 0.8% Ultroser G added; the cells were then counted and diluted to 50,000/100 µL. One hundred microliters of this cell suspension was added to each well coated with ECM in a 96-well multiwell plate, followed by 100 µL of the appropriate dilution of toxin. For each dilution series of soluble toxins a control culture was set up containing 50,000 cells which were not exposed to toxin. In each dilution series of lipophilic agents, a second control culture containing 50,000 cells exposed to 0.5% methanol was included. The cultures were maintained at 37°C in 95% air and 5% CO₂ for a period of 20 hr. After this time the medium and unattached cells were removed by washing the cell sheet with phosphate-buffered 0.15 M NaCl. The remaining cells attached in control cultures and those exposed to toxins were stained for NBT. The mean number of attached cells in the field of view of test cultures (T) and control (C₁ or C₂, where 1 = no additions and 2 = 0.5% methanol) was determined. By considering the attachment of control cultures as 100% the relative attachment of the cells in the toxin-treated cultures were calculated as T/C₁ or C₂ \times 100%. The attachment efficiency of functionally competent Clara cells that were exposed to different concentrations of toxins was plotted against the concentration of toxin used. Thus, for each toxin a TD₅₀ value (the concentration of toxin to reduce attachment by 50% of control levels) could be calculated.

**Results and Discussion**

**The Effects of Different Media and Substrata on the Attachment of Clara Cells In Vitro**

While 15 to 20% of the original population of NBT positive Clara cells attached to a plastic substratum over 22 hr in the presence of 10% fetal bovine serum (plus Ham's F12) or the serum substitute (2% Ultroser G plus Ham's F12), the cells did not attach on plastic alone when maintained in Ham's F12 that was supplemented with hormones and growth factor (Fig. 1a). Similar results were found when the plastic substratum was coated with...
BSA or fibronectin and the cells were examined at 22 hr or 40 hr in culture (Figs. 1a and b). However, when the cells were placed in multiwells coated with Vitrogen or a Vitrogen/fibronectin combination, some Clara cells did attach in F12/H medium. Thus, attachment in this case does not require the protein components (and other unknown factors) present in serum or serum substitute. It would therefore appear that collagen (and any attached proteoglycan/glycoproteins) has an important role in the attachment of Clara cells. Indeed, in subsequent experiments, a good attachment and the spreading of NBT positive cells were obtained when Type IV collagen was substituted for vitrogen and the cells were maintained in 2% Ultroser G plus F12 (Fig. 2).

**Attachment and Spreading of Clara Cells on Extracellular Matrix**

As noted in the previous experiments, approximately 15% of a starting population of NBT positive Clara cells attach to plastic in the presence of 2% Ultroser G plus F12 over a 20 hr period (Fig. 3a). Few cells attach to plastic in F12/H (hormone and growth factor supplemented) medium or in the single strength serum substitute medium, DCCM1, which contains protein (bovine albumin, fraction 5) but no epidermal or epithelial growth factors. Clara cells attach well to plastic when these media (F12/H or DCCM1) are supplemented with a small amount of fetal bovine serum (2%) or Ultroser G (0.4%). The importance of the extracellular matrix (ECM) in the attachment efficiency, irrespective of the culture medium used, is clearly shown at 20 hr (Fig. 3a). Between 45% and 70% of the starting population of NBT positive Clara cells attach to the ECM substratum. It appears that the attachment efficiency of Clara cells is reduced by 42 hr in culture (Fig. 3b). However, more total cells (Clara and other cell types) attach at 42 hr, but many have lost the ability to stain positively with the NBT. It is noticeable that the loss of NADPH-cytochrome (P-450) reductase activity (NBT staining) is not as extensive in cultures that contain F12/H medium.

Apart from attachment efficiency, the manner in which Clara cells flatten and spread in culture is found to be dependent on both the culture medium and the substratum (Table 2). Thus, the best spreading on plastic surfaces (considered moderately good) is found

![Figure 2](image_url)
with 2% fetal bovine serum plus DCCM1 at 20 hr. Limited (poor) spreading on a plastic substratum is noted with Ultroser plus F12, Ultroser plus DCCM1, or 2% fetal bovine serum plus F12/H. The few cells that do attach to plastic when maintained in F12/H or DCCM1 remain totally rounded. Similarly, Clara cells maintained in 0.4% Ultroser plus F12/H for 20 hr do not spread on plastic (Table 2), despite the fact that a large number of cells manage to attach to the substratum (Fig. 3a).

**Effects of Bronchiolar Toxins on the Plating Efficiency of Clara Cells**

**Culture Conditions and the Toxicity Assay.** Fresh isolates of Clara cells were equilibrated in DCCM1 medium for 2 hr at 37°C in plastic Petri dishes. The unattached Clara cells, diluted to 40,000 to 50,000/100 μL of DCCM1, were mixed with the appropriate concentration of toxic agent and cultured on ECM in a multiwell plate in a final concentration of 0.4% Ultroser plus DCCM1 for a further 20 hr. From the studies described previously, this combination of medium and substratum was considered the best for attachment of a large number of cells, the cells also spread reasonably well within the 20 hr and retained a high level of functional competence as determined by NBT staining. After 20 hr approximately 30 to 40% attachment efficiency was found in control cultures (no toxin added), and the majority of cells were very well spread on the ECM substratum (Fig. 4a) making enumeration easy.

For many of the compounds investigated a second control culture (solvent control) was also included to assess the effect of the solvent (usually methanol, final concentration 0.5%) on attachment efficiency. This solvent had very little effect on attachment efficiency of NBT positive Clara cells. If the attachment efficiency in control (toxin and solvent free) cultures was taken as 100% then the addition of methanol (final concentration 0.5%) reduced the attachment efficiency to 92 ± (SD) 12 (n = 16). Dimethylformamide (0.5%) reduced attachment efficiency to 85% (control = 100%, n = 1) but ethanol was without effect. None of the solvents used has any effect on the spreading characteristics of the cells.

**Reproducibility of the Toxicity Assay Using Paraquat.** When Clara cells were exposed to 10⁻² M paraquat for 20 hr there was no extensive cell death, and the attachment efficiency of the cells remained high (Fig. 4c). However, there was some loss of NBT staining and a number of cells appeared to be discharging a portion of NBT-positive cytoplasm (apical bud?) that was often connected to the main portion of the cell by a thin strand (Fig. 4b). Cultures exposed to 10⁻⁶ M paraquat (Fig. 4d) can have many of these free apical buds, although some cells remained spread and retained positive NBT staining. With increasing concentrations of paraquat (10⁻⁵ M, 10⁻⁴ M; Figs. 4e and f), attachment efficiency and spreading was reduced. Cells staining for NBT were counted in control and paraquat-treated cultures. The attachment efficiency of functionally competent Clara cells in the herbicide-treated cultures was plotted as a percentage of the control culture (taken as 100%) (Fig. 5). From the results of repeating this experiment with four separate isolates of Clara cells, the mean TD₅₀ value (the concentration of paraquat required to reduced normal attachment efficiency by 50%) was found to be 3.7 ± (SD) 2.8 μM (Fig. 5).

**Toxicity Rating of Different Compounds.** The effects of a total of 26 compounds on the attachment efficiency profiles of Clara cells are shown in Figure 6. The TD₅₀ values, calculated from this data (Fig. 6) are plotted in Figure 7 and each compound given an arbitrary toxicity rating as very high (TD₅₀ < 1 μM), high (1–10 μM), moderately high (11–100 μM), weak (101–1000 μM), or minimal (> 1000 μM).

One surprising result is the apparent weak or minimal toxicity of the halogenated hydrocarbons in that TD₅₀ values are not obtained for trichloro- or dichloroethylene; 1,2-; 1,3-dichlorobenzene or bromobenzene. In addition, the TD₅₀ value for chlorobenzene (900 μM) is high and both 1,3- and 1,4-dichlorobenzene are equally toxic at 10 μM as they are at 1000 μM (Fig. 6). Most of the halogenated hydrocarbons undergo biotransformation by P-450 enzymes to form epoxides that are considered to be the toxic metabolite (6). These can be
Figure 4. (a) Clara cells cultured for 20 hr on ECM substratum and in a medium of DCCM1 plus 0.4% Ultroser. Most of the cells are well spread but not all are intensely stained with the NBT. ×540. (b) Cells as for (a) but treated with $10^{-7}$ M paraquat. Note the loss of NBT staining and the loss of apical cytoplasmic buds (arrow). ×540. (c-f) Cells as for (a) but treated with $10^{-7}$ M, $10^{-6}$ M, $10^{-5}$ M, $10^{-4}$ M paraquat, respectively. ×140.
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Figure 5. The effect of paraquat on the attachment efficiency of functionally competent Clara cells maintained in ECM-coated wells in DCCM1 and 0.4% Ultroser. The data are from four separate isolations and the TD₅₀ value for each experiment is shown.

Figure 6. The effect of 26 different agents on the attachment efficiency of functionally competent Clara cells maintained in DCCM1 plus 0.4% Ultroser on ECM-coated wells for 20 hr.

detoxified by epoxide hydrolase to form the corresponding diol metabolites or degraded through the action of glutathione transferase or other phase II enzymes. One exception is the epoxide derived from 1,4(p)-dichlorobenzene that is considered too unstable to react with glutathione. Interestingly, the 1,4-dichlorobenzene, even when given in high doses IP, is reported not to induce Clara cell necrosis (4) and, yet, this compound is the most effective of all the hydrocarbons in reducing attachment efficiency in Clara cell cultures in vitro (Fig. 7).

Most of the other halogenated hydrocarbons have been claimed to produce Clara cell necrosis when administered IP (4), although it is unclear whether or not the parent compounds target directly to the lung cells or that primary P-450 metabolism occurs in the liver and the metabolites that are produced are responsible for Clara cell damage. Thus, it is possible that Clara cells do not possess the specific P-450 isozymes necessary for the first steps of halogenated hydrocarbon biotransformation. Equally, it is possible that Clara cells in vitro have lost the specific P-450 enzyme as a result of the isolation procedure or that the enzyme is not inducible under the conditions of cell culture. A third possibility relates to the technical design of the experiments in that some of the hydrocarbons are volatile. This applies particularly to dichloroethylene, and thus the Clara cell cultures may not be receiving the expected level of exposure. None of these suggestions offer an entirely satisfactory explanation for the apparent low toxicity of the halogenated hydrocarbons, and further studies with adap-
tations to the culture system will be necessary to investigate the effects of these compounds.

Studies have established that freshly isolated mouse Clara cells contain both monoxygenase activity (24) and contain epoxide hydrolase (Richards, Oreffo, and Lewis, unpublished data). Thus, it is interesting that another hydrocarbon, naphthalene, is moderately toxic (TD$_{50}$ value 110 $\mu$M) to Clara cells in culture. This aromatic hydrocarbon is converted to a reactive arene oxide by the P-450 system, and either the parent compound or the metabolite(s) selectively initiates bronchiolar Clara cell necrosis in mice (7). Two other compounds, 4-ipomeanol and 3-methylindole, which also produce extensive Clara cell necrosis in mice (8–II), are very highly toxic (TD$_{50}$ value < 0.1 $\mu$M) and highly toxic (TD$_{50}$ = 5 $\mu$M), respectively, to Clara cell cultures (Figs. 6 and 7). P-450 mediated oxidation of the furan ring of 4-ipomeanol yields reactive metabolites that bind to Clara cell proteins, thus, enhancing cell necrosis (8). Methylindole may not require activation to induce toxicity, but the compound is detoxified by P-450 enzymes and glutathione conjugation. Other agents that are thought to require P-450-mediated activation to produce reactive, carcinogenic metabolites in vivo are also investigated in the Clara cell culture. These include nitrosodiethylnime (TD$_{50}$ in Clara cell cultures = 100 $\mu$M), urethane (TD$_{50}$ = 900 $\mu$M), and methylcholanthrene (TD$_{50}$ > 100 $\mu$M). Mouse Clara cells in vitro are also moderately damaged by butylated hydroxytoluene (TD$_{50}$ = 90 $\mu$M). Mice are known to be highly sensitive to this compound (16) that may require P-450 activation to act as a promoter of lung tumor formation.

The anticancer drug cyclophosphamide has not been reported to damage Clara cells in vivo, and the freshly purchased (newly purchased) sample used shows minimal toxicity to Clara cells in culture. However, an aged sample of cyclophosphamide, maintained at room temperature for 5 years, may be expected to have undergone some degradation, and, indeed, this sample is moderately toxic (TD$_{50}$ = 60 $\mu$M) to Clara cells in vitro. Acrolein, a degradation product of cyclophosphamide (16), is also moderately toxic (TD$_{50}$ = 100 $\mu$M) in the in vitro system. Another drug, busulfan, has little effect on Clara cell attachment efficiency at doses < 100 $\mu$M, a finding that is similar to that with alloxan, an agent considered to selectively damage lung endothelial cells (18).

The herbicide paraquat has been shown to be actively accumulated by lung tissue and is considered to target to epithelial (Type I and II) cells (16,19). This diamine also causes extensive changes to Clara cells in vitro (18) and has a high toxicity rating (TD$_{50}$ = 4 $\mu$M) from the present in vitro studies.

Diquat, like paraquat, does not require P-450 activation but probably damages cells as a result of redox cycling. Diquat, however, is not accumulated by the lung (19) (and thus by inference is not accumulated by lung epithelial cells) and is therefore less toxic than paraquat in vivo. However, toxicity studies with rat Type II cells maintained in vitro have shown that diquat is eight times more damaging than paraquat (21). It is therefore interesting to note from the present investigation that at least 10 times as much diquat (TD$_{50}$ = 45 $\mu$M) is required to reduce attachment efficiency of Clara cells in culture by the same amount as that noted for paraquat. The fact that Clara cells are highly sensitive to paraquat does fit well with the fact that these cells are a prime target for the herbicide in vitro. The endogenous polyamine spermidine produces damage to Clara cells when administered by inhalation to rats and is moderately toxic (TD$_{50}$ = 80 $\mu$M) in the present in vitro study with mouse Clara cells.

Both cadmium and hexavalent chromium produce potent edematous reactions in lung tissue and are well-established cell damaging agents. It is therefore not surprising that they are both highly toxic (TD$_{50}$ = 2 $\mu$M and 5 $\mu$M, respectively) in Clara cell cultures. In contrast, nickel chloride is without effect and does not extensively damage rat Type II cells maintained in vitro (21). Zinc chloride and the endothelial cell toxin ammonium sulfate (29) at concentrations > 10 $\mu$M appear to promote the attachment efficiency of NBT-positive Clara cells above that noted in control cultures. This possibility that zinc

![Figure 8](image-url)
chloride or ammonium sulfate may assist in reducing the loss of NADPH-cytochrome P-450 reductase activity or is instrumental in promoting Clara cell attachment appears worthy of further investigation.

The relationship between the TD50 values obtained with Clara cells exposed to chemicals in vitro was compared, wherever possible, with LD50 values obtained following oral intake of these same chemicals by rodents (Table 1). A double log plot and regression analysis (Fig. 8) indicated an interesting relationship between the values obtained for 13 compounds. The Clara cells in vitro were more sensitive to the chemicals that had the lowest oral LD50 values in rodents (mostly mouse) in vivo. With the possible exception of 1,2-dichlorobenzene, which has a low LD50 of 500 mg/kg body wt (oral in the rat), the correlation between the simple in vitro system described and the LD50 values in vivo is reasonably good, especially in view of the fact that death from the oral intake of a chemical may not necessarily result from lung damage.

It is certainly premature to advocate the use of Clara cell cultures to screen chemicals likely to cause pulmonary damage. Nevertheless, the basic study described here warrants further investigation. In addition, the simple system described will permit a study of the effects of combinations of chemicals on Clara cells much more easily than can be carried out in vivo.

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