Characterization of Clara and Type II cells isolated from rat lung by fluorescence-activated flow cytometry

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A novel procedure for the isolation of Clara cells from rat lung is described. Single-cell suspensions from male F344/TOX rat lungs, prepared by subtilisin digestion, were treated with monochlorobimane (10 μ M) and analysed by fluorescence-activated flow cytometry. A sub-population of about 2.8% of the total, showing the highest blue fluorescence and by inference the highest GSH concentration, was isolated as Clara cells of > 95% purity. Type II cells of similar purity were sorted after treatment with Phosphine 3R. Both sub-populations were > 90% viable, as judged by Trypan Blue exclusion. Comparison of CYP (cytochrome *P*-450) isoenzymes between these sub-populations, using Western blotting, showed CYP1A1 to be barely detectable. In Clara cells, CYP2B1 was 10-fold higher than in Type II cells. Mono-oxygenase activity towards the *O*-deethylation of 3-cyano-7-ethoxycoumarin was 2-fold higher in

Clara cells. No activity was detected in macrophages. Pretreating rats with the mono-oxygenase inducers phenobarbitone, 3-methylcholanthrene or Aroclor 1254 showed the last-named to be the most potent inducer of CYP1A1. In Clara cells, CYP1A1 concentration and mono-oxygenase activities were induced > 2000- and 50-fold respectively, whereas in Type II cells increases of 300- and 3.6-fold were seen. Clara cells isolated from the lungs of control rats had a concentration of GSH (2.7 nmol/10⁶ cells) that was 9- and 2-fold higher than that in Type II cells or macrophages respectively. GSH depleted by monochlorobimane treatment was restored after 2–3 h incubation with 0.2 M *N*-acetylcysteine. γ -Glutamyltranspeptidase activity in Clara cells was 6- and 50-fold higher than in Type II cells or macrophages respectively.

INTRODUCTION

Many pulmonary toxins and chemical carcinogens need to undergo bioactivation in rat lung [reviewed in (1)]. Their cytotoxic or tumorigenic action is often exerted in only some of the over 40 different cell types present [2], with the non-ciliated bronchiolar epithelial (Clara) cells, Type I and Type II cells often being the most severely affected [3]. Differences in susceptibility may be associated with differences in the activity of the bioactivating enzymes, particularly the CYP (cytochrome *P*-450) mono-oxygenases [3,4], Phase II detoxifying systems and the intracellular concentration of GSH (reviewed in [5]). Immunohistochemistry has been employed extensively to demonstrate the differential intracellular localization of the CYP mono-oxygenases in lung sections [6,7]. The use of isolated purified sub-populations of lung cells for such studies has been limited, particularly for Clara cells from rat lung, which represent < 2% of the total [8]. Isolation by the technique of centrifugal elutriation resulted in a purity of only 60% [8]. It has been shown that Clara cells isolated in this way have 1.5–4-fold higher mono-oxygenase activity than Type II cells and that this activity can be increased by pretreatment of rats with the inducer β -naphthoflavone [8].

Concentrations of GSH in Clara cells may be 2–3-fold higher than those of Type II cells [9]. Immunolocalization of γ -glutamyltranspeptidase on the apical surfaces of Clara cells in sections of rat lung have suggested a homeostatic role for this cell type [10], utilizing the high levels of GSH in the alveolar epithelial lining fluid [11].

We have developed a novel procedure for the isolation of Clara cells of high purity from rat lung using fluorescence-activated flow cytometry. The aim of the present study was to characterize the expression and the activity of their cytochrome

P-450-dependent mono-oxygenases, to compare the inducibility relative to Type II cells and macrophages and to determine the relative levels of both GSH and γ -glutamyltranspeptidase in these different cell types.

MATERIALS AND METHODS

Chemicals

Subtilisin (EC 3.4.21.62; 5 units/mg) was from Boehringer, Lewes, E. Sussex, U.K. Sagatal was from RMB, Dagenham, Essex, U.K. Aroclor 1254 was from Monsanto, St. Louis, MO, U.S.A. Monochlorobimane, monobromobimane, diethoxyfluorescein and 3-cyano-7-ethoxycoumarin were from Molecular Probes, Eugene, OR, U.S.A. Phosphine 3R was from Phase-Sep, Deeside, Clwyd, U.K. Solutions for perfusion and tissue-culture media were obtained from GIBCO, Paisley, Scotland, U.K. CYP antigens and polyclonal antibodies were generously given by Professor C. R. Wolf of ICRF, Dundee, Scotland, U.K. All other chemicals were from Sigma Chemical Co., Poole, Dorset, U.K., and were of the highest available quality.

Animals

Male F344/TOX rats (170–190 g) were bred on site. On various occasions animals were pretreated via the intraperitoneal route with the following compounds: Aroclor 1254, administered as a single dose of 200 mg/kg (200 mg/ml solution in trioctanoin), 3 days before being killed; 3-methylcholanthrene, three doses of 20 mg/kg (10 mg/ml dissolved in trioctanoin), at 24 h intervals before being killed on day 4; or phenobarbitone, three doses of 100 mg/kg (100 mg/ml dissolved in 0.14 M NaCl) at 24 h intervals before being killed on day 4.

Abbreviations used: CYP, cytochrome *P*-450; HBSS, Hanks buffered saline solution.

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Perfusion procedures

Enzymic dispersion of rat lungs followed the procedures previously described [12]. Rats were anaesthetized with Sagatal (250 mg/kg intraperitoneally) and the lungs were cleared of blood by perfusion through the pulmonary artery with oxygenated Hanks buffered saline solution (HBSS). Macrophages were removed through a tracheal cannula by using 3 × 4 ml of phosphate-buffered saline. The heart and lungs were removed and the lungs were perfused for 20 min with subtilisin (0.17 mg/ml) in HBSS at 37 °C gassed with O₂/CO₂ (19:1) in a recirculating perfusion apparatus. The lungs were removed, shredded by using two pairs of fine forceps, dispersed in HBSS containing foetal-bovine serum (10%, v/v) and DNAase (5 mg/ml) (supplemented HBSS) and filtered through a 125 µm-mesh nylon gauze. Cells were washed with 2 × 5 ml of supplemented HBSS and resuspended in supplemented HBSS giving a recovery of $(4 \pm 0.16) \times 10^7$ cells (mean ± S.E.M., 10 experiments) and a viability of > 90%, as judged by Trypan Blue staining and counting in a haemocytometer cell.

Fluorescent labelling of cells: flow cytometry

(a) Clara cells

To a suspension of rat lung cells (1×10^7), in supplemented HBSS (10 ml) at 37 °C, was added monochlorobimane (final concn. 10 µM, dissolved in 10 µl of CH₃CN). The mixture was incubated in the dark for 5 min at 37 °C. The cells were centrifuged (300 g for 4 min at 20 °C) and resuspended in Leibovitz L-15 medium supplemented with foetal-bovine serum (5%, v/v), L-glutamine (1%, w/v), DNAase (5 mg/ml) and gentamycin (50 µg/ml). Fluorescence-activated cell sorting was carried out with an Ortho 50H Cytofluorograf instrument with a Data General 2150 computer system (Ortho Diagnostic Systems, Westwood, MA, U.S.A.). The 407 nm line of a krypton-ion laser was used for fluorescence excitation, and emission was determined by using a 430 nm long-pass filter. Narrow-angle (2°) forward and side (90°) scatter were recorded simultaneously. Clara cells were sorted at a rate of approx. 50/s into 0.2 ml of supplemented Leibovitz L-15 medium.

In some experiments, where rats had been pretreated with Aroclor 1254 as described above to induce drug-metabolizing enzyme systems, the isolated lung cell preparation (1×10^7 cells) was incubated for 5 min at 37 °C in supplemented HBSS (10 ml) containing 0.03 mM diethoxyfluorescein. Cells were recovered by centrifugation (300 g for 4 min) and resuspended in supplemented Leibovitz L-15 medium (10 ml). Fluorescence-activated flow cytometry was carried out by using the 488 nm line of an argon-ion laser as the excitation wavelength and a 530 nm band-pass emission filter. Cells which had metabolized diethoxyfluorescein to fluorescein were sorted at a rate of about 30/s.

(b) Type II cells

Lung cells (1×10^7) in supplemented HBSS (10 ml) were incubated in the dark at 20 °C with Phosphine 3R (final concn. 1 µM, dissolved in 10 µl of dimethyl sulphoxide) as described by Leary et al. [13], but for only 5 min. The cells were centrifuged (300 g for 4 min at 20 °C) and resuspended in supplemented Leibovitz L-15 medium as described above. For flow cytometry, the 488 nm line of an argon-ion laser was used for fluorescence excitation. Emission was determined by using a 530 nm band-pass filter. Sorting was carried out at a rate of about 100 cells/s.

(c) Macrophages

Macrophages (1×10^6 cells) from lung lavage were suspended in supplemented Leibovitz L-15 medium (5 ml) and sorted on the basis of their forward versus 90° light scatter by using the 488 nm argon-ion laser. Sorting was carried out at a rate of approx. 300 cells/s.

In all instances, the sorting efficiency of the flow cytometer was checked by counting a sample of the sorted population in a Coulter counter (Channelizer 256; Coulter Electronics, Luton, Beds., U.K.). Because of the slow sorting rate, separate animals were used for the preparation of each cell type.

SDS/PAGE

Electrophoresis was carried out on a 3% (w/v) stacking/9% (w/v) running gel [14]. Solubilized lung cells (50000), in sample buffer, were applied in a volume of 10 µl. After electrophoresis, proteins were transferred electrophoretically to a nitrocellulose membrane. The nitrocellulose sheets were blocked with 3% (w/v) defatted milk protein for 1 h. Sheets were incubated first with the appropriate anti-CYP antibody (1:500 dilution) and subsequently with an alkaline-phosphatase-conjugated anti-rabbit antibody (1:1000 dilution). All reactions were carried out at 20 °C. Immunoreactive bands were revealed by incubation with Nitro Blue Tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate [15]. Reactions were stopped by rinsing the sheets in phosphate-buffered saline containing 20 mM EDTA. The density of the bands was determined by means of a soft-laser scanning densitometer and recording integrator (LKB 2202 Ultrascan; Pharmacia, Milton Keynes, Bucks, U.K.). Quantification was performed relative to CYP standards (0.2–1 pmol) processed in a similar manner. Within this range, the area recorded by the integrator was directly proportional to the amount of the CYP standard.

O-De-ethylation of 3-cyano-7-ethoxycoumarin

The O-deethylation of 3-cyano-7-ethoxycoumarin to 3-cyano-7-hydroxycoumarin was determined fluorimetrically [16]. Reaction mixtures of 3 ml volume in a stirred fluorimeter cuvette at 37 °C contained: KCl, 1.15% (w/v); EDTA, 5 mM; MgCl₂, 10 mM; and 0.1 M Hepes/NaOH buffer, pH 7.4. Freshly isolated unsorted cells (1×10^6) or sorted sub-populations (1×10^6 cells) were added, followed by 3-cyano-7-ethoxycoumarin (final concn. 25 µM, dissolved in 8 µl of dimethyl sulphoxide). Fluorescence was recorded continuously by using excitation and emission wavelengths of 405 and 455 nm respectively (LS5 fluorimeter; Parkin-Elmer, Beaconsfield, Bucks., U.K.). In some instances NADPH (0.2 mM) was added to the reaction mixture after cells had been frozen and thawed (twice) in liquid N₂.

Electron and light microscopy

Sorted cells (1×10^5) for transmission electron microscopy were fixed in glutaraldehyde (2%, v/v), treated with OsO₄ and embedded in Araldite. Ultrathin sections were stained with uranyl acetate and lead citrate before examination in a Jeol 100-CX ASID electron microscope (Jeol U.K. Ltd., Colindale, London, U.K.). For light-microscopical examination, 1 µm semi-thin sections were cut from the same samples and stained with Toluidine Blue.

Determination of GSSG and GSH in isolated lung cells

This was done by h.p.l.c. after derivative formation with dansyl chloride [17]. Unsorted cells (1×10^6) or sorted sub-populations

Figure 1 Fluorescence-activated analysis of lung cells after incubation with monochlorobimane

Isolated rat lung cells (10^7) were incubated in supplemented Hanks medium (10 ml) containing DNAase (5 mg/ml) and monochlorobimane (10 μ M) for 5 min at 37 °C in the dark. After centrifugation and resuspension, cells were subjected to fluorescence-activated analysis using an excitation wavelength of 407 nm; emission filter > 430 nm. Panel (a) represents total blue fluorescence of the unsorted population. The most fluorescent cells are selected as region R1. Panel (b) shows the forward-versus-90° scatter of the cells defined by region R1. The scatter properties are defined by the area R2, which is transferred electronically as R3 to panel (c), which shows the forward-versus-90° scatter for the unsorted lung cell population. The fluorescence properties only from R3 are displayed in panel (d). The most fluorescent subpopulation is selected as region R4 and sorted to give Clara cells of 95% purity.

(1×10^5 cells) were dispersed in 400 μ l of ice-cold HClO_4 (5%, v/v) containing boric acid (0.2 M), diethylenetriaminepenta-acetic acid (2 mM) and Cresol Red indicator (0.05 mg/ml). After centrifugation (10000 g for 4 min at 4 °C), the supernatant was removed and the derivative was prepared with iodoacetic acid and dansyl chloride as described previously [17].

Estimation of GSH/monochlorobimane adducts by h.p.l.c.

After sorting, cells (1×10^5) were recovered by centrifugation (300 g for 4 min at 20 °C) and dispersed through a pipette tip with ice-cold HClO_4 (50 μ l of a 5% solution). Protein was precipitated (10000 g for 4 min at 4 °C), and a portion of the supernatant (20 μ l) was immediately subjected to reverse-phase h.p.l.c. [18,19]. Separation of the GSH/monochlorobimane adduct was performed on a 5 μ m LiChrospher C_{18} column (12.5 cm $\times$ 0.4 cm; E. Merck, Darmstadt, Germany) fitted with a pre-column (0.4 cm $\times$ 0.4 cm) packed with 45 μ m pellicular ODS silica. A solvent system of $\text{CH}_3\text{CN}/\text{H}_2\text{O}$ (1:20, v/v) to $\text{CH}_3\text{CN}/\text{H}_2\text{O}$ (4:1, v/v) was used as previously described [19].

Both mobile phases contained acetic acid (0.25%, v/v) and NaClO_4 (0.7%, w/v). A linear gradient over 15 min was used at a flow rate of 1.3 ml/min. The effluent was monitored by a fluorescence detector (Waters 470, Millipore, Watford, Herts., U.K.) with the excitation and emission wavelengths set to 393 and 475 nm respectively. Standards were prepared by chemically treating GSH (0–10 nmol) with monobromobimane (100 nmol, dissolved in 2 μ l of CH_3CN) in 0.05 M Hepes/NaOH buffer, pH 8.0 (500 μ l), for 20 min in the dark at 20 °C. Reactions were stabilized by addition of acetic acid (10 μ l) and the products were immediately analysed by h.p.l.c. as described above.

γ -Glutamyltranspeptidase

Analysis of γ -glutamyltranspeptidase activities was based on the fluorimetric method of Smith et al. [20]. Rat lung cells (1×10^5) were resuspended in a reaction volume of 350 μ l of 2-amino-2-methylpropane-1,3-diol/HCl buffer, pH 8.5, containing glycylglycine (20 mM) and Triton X-100 (0.1%, w/v) at 37 °C. Reactions were started by the addition of γ -glutamyl-7-amino-4-

Figure 2 Light micrographs of (a) Clara cells separated by flow cytometry after their reaction with monochlorobimane as described in the legend to Figure 1, and (b) Type II cells separated by flow cytometry after staining with Phosphine 3R as described in the Materials and methods section

Cells were fixed in glutaraldehyde, treated with OsO_4 , and semi-thin sections were stained with Toluidine Blue. Bars represent 10 μm .

methylcoumarin (final concn. 0.14 mM, dissolved in 10 μl of 2-methoxyethanol). After incubation for 10 min at 37 °C, reactions were stopped by addition of ice-cold 50 mM glycine/NaOH buffer, pH 10.4 (2 ml). The fluorescence was determined immediately by using excitation and emission wavelengths of 370 and 440 nm respectively.

Synthesis of intracellular GSH and loss of GSH/monochlorobimane adducts in purified sub-populations of Clara cells

Sorted Clara cells (1×10^5) were maintained in 0.5 ml of Dulbecco's modified Eagle's medium supplemented with foetal-bovine serum (5%, v/v), glutamine (1%, w/v) and gentamycin (5 $\mu\text{g}/\text{ml}$), to which was added freshly prepared *N*-acetylcysteine (0.2 mM) immediately before use. Cells were cultured in suspension at 37 °C in 5% CO_2 in air for up to 4 h after sorting. After incubation, cells were recovered by centrifugation (300 *g* for 4 min), and the cell pellet was analysed for GSH and GSSG or GSH/monochlorobimane-adduct concentrations by the h.p.l.c. procedures described above.

RESULTS

Isolation of purified rat lung cell sub-populations by fluorescence-activated flow cytometry

(a) Clara cells

When rat lung cells were incubated for 5 min with monochlorobimane (10 μM) and examined by u.v. fluorescence microscopy, about 2% of the population showed a strong blue fluorescence, compared with the weaker fluorescence exhibited by many of the remaining cells. Fluorescence-activated analysis of lung cells treated in this way showed the presence of two sub-populations separated by their blue fluorescence properties (Figure 1a). The more strongly fluorescent population (region

R1) was defined and the forward-versus-90° scatter properties of these cells are shown in Figure 1(b). A computer-generated region (Figure 1b, region R2) was defined and electronically transferred to the forward-versus-90° scatter pattern of the whole population (Figure 1c, region R3). The fluorescence characteristics of cells with these scatter properties are shown in Figure 1(d). A region of the most highly fluorescing cells was defined (region R4) and sorted to give a sub-population ($2.8 \pm 0.3\%$ of the total; mean $\pm$ S.E.M., $n = 9$) of viability $95 \pm 1\%$ (mean $\pm$ S.E.M., $n = 9$), as judged by Trypan Blue staining. Freshly isolated cells had a diameter of $10.5 \pm 1.2 \mu\text{m}$ (mean $\pm$ S.D.; Coulter Channelizer). When examined by light and electron microscopy (Figures 2a and 3a), the preparation was shown to be non-ciliated epithelial (Clara) cells, as judged by the presence of extensive smooth endoplasmic reticulum, secretory granules and indented nuclei, and was of > 95% purity. Some cells also showed vacuolation to a varying degree, probably as a consequence of the enzymic digestion procedures. The characteristic features of a Clara cell are seen more clearly by transmission electron microscopy (Figure 3a), especially the abundance of smooth endoplasmic reticulum and the electron-dense secretory granules. An intact nuclear membrane and a mitochondrion with well-defined cristae can also be seen. Clara cells isolated in this way formed monolayers on tissue-culture plates coated with an extracellular matrix (Biological Industries, Cumbernauld, Glasgow, U.K.) when cultured for 24 h in the presence of 0.2 mM *N*-acetylcysteine (results not shown).

To investigate whether the mono-oxygenase activity of Clara cells could be used as a basis for separation, lung cells from rats pretreated with Aroclor were incubated with diethoxyfluorescein (0.03 mM) for 5 min at 37 °C and analysed by flow cytometry. A pattern of green fluorescent cells similar to that shown in Figure 1 was seen. Isolation of these cells and examination by electron microscopy showed them to be Clara cells of > 95% purity.

Figure 3 Transmission electron micrographs of (a) a Clara cell prepared as described in the legend of Figure 2(a), except that ultra-thin sections were stained with uranyl acetate, and (b) a Type II cell prepared as described above

In (a), arrows indicate smooth endoplasmic reticulum, and arrowheads secretory granules. In (b) arrows indicate lamellar bodies, and the arrowhead shows multi-vesicular bodies. Bars indicate 1 μm .

However, no Clara cells could be isolated by this technique when preparations from control rats were used.

(b) Type II cells

Type II cells were isolated by staining with Phosphine 3R (1 μM) and fluorescence activated sorting as described by Leary et al.

[13]. A light micrograph (Figure 2b) of these sorted cells shows a population of Type II cells, although the lamellar bodies have largely been lost in this particular preparation. Such cells represented $12.2 \pm 2\%$ (mean $\pm$ S.E.M., $n = 5$) of the unsorted population and were of $> 95\%$ purity. Their diameter was $9.1 \pm 0.8 \mu\text{m}$ (mean $\pm$ S.E.; Coulter Channelizer) and they had a viability of $90 \pm 2\%$ (mean $\pm$ S.E.M., $n = 9$). Their lamellar

Table 1 *O*-De-ethylation of 3-cyano-7-ethoxycoumarin by sub-populations of rat lung cells

Cells were obtained by subtilisin digestion of lungs from control male rats or those that had been pretreated with Aroclor 1254, phenobarbitone or 3-methylcholanthrene as described in the Materials and methods section. Clara and Type II cells were isolated by fluorescence-activated flow cytometry after treatment with either monochlorobimane or Phosphine 3R respectively. Cell samples (10^5) were incubated at 37 °C in a stirred fluorimeter cuvette with 3-cyano-7-ethoxycoumarin (25 μ M), and the increase in fluorescence was continuously monitored by using excitation and emission wavelengths of 405 and 455 nm respectively. Initial reaction rates were calculated relative to a 3-cyano-7-hydroxycoumarin standard curve. Results represent means $\pm$ S.E.M. (number of experiments). Probability of significance in activity between Clara and Type II cells: *** P < 0.001, ** P < 0.01; NS, not significant.

Pretreatment	3-Cyano-7-hydroxycoumarin formation (pmol/min per 10 ⁶ cells)			
	Cells...	Unsorted	Clara	Type II
Control		9 ± 0.9 (7)	58 ± 15 (3)	25 ± 13 (4) NS
Aroclor 1254		192 ± 34 (9)	924 ± 112 (5)	90 ± 8.9 (3)***
Phenobarbitone		11 ± 2.2 (7)	64 ± 12 (3)	16 ± 3.8 (4)**
3-Methylcholanthrene		65 ± 20 (8)	205 ± 73 (4)	27 ± 0.9 (4) NS

Table 2 CYP1A1 and CYP2B1 in Clara and Type II cells from rat lung

Purified sub-populations of lung cells were obtained by flow cytometry from control or pretreated rats as described in the legend to Table 1. Cells (5×10^4) were run on SDS/9% (w/v)-polyacrylamide gels. After electrophoresis, proteins were transferred to nitrocellulose sheets. CYP proteins were detected by the use of the appropriate antibody raised in rabbits, followed by an anti-rabbit alkaline phosphatase conjugated second antibody and incubation with Nitro Blue Tetrazolium/5-bromo-4-chloro-3-indolyl phosphate. Concentrations of CYP were determined by laser densitometry relative to cytochrome *P*-450 standards. Results represent means $\pm$ S.E.M. (4 experiments). Probability of significance in concentration between Clara and Type II cells: ** P < 0.01, * P < 0.05; NS, not significant.

Pretreatment	Cells...	CYP isoenzyme (pmol/10 ⁶ cells)		
		Unsorted	Clara	Type II
(a) CYP1A1				
Control		0.09 ± 0.032	0.012†	0.004†
Aroclor 1254		3.60 ± 0.7	25 ± 5.2	1.2 ± 0.38**
Phenobarbitone		0.24 ± 0.15	‡	‡
3-Methylcholanthrene		3.18 ± 0.47	3.8 ± 1.3	1.2 ± 0.48 NS
(b) CYP2B1				
Control		1.30 ± 0.44	3.1 ± 1.2	0.30 ± 0.13 NS
Aroclor 1254		0.88 ± 0.21	1.5 ± 0.4	0.44 ± 0.13*
Phenobarbitone		1.10 ± 0.35	1.3 ± 0.5	0.40 ± 0.23 NS
3-Methylcholanthrene		0.87 ± 0.20	1.9 ± 0.7	0.36 ± 0.12 NS

† Mean of 2 experiments.
‡ Below limit of detection.

† Mean of 2 experiments.

‡ Below limit of detection.

bodies can be seen in a transmission electron micrograph (Figure 3b). An intact subcellular structure is evident, including mitochondria and a multi-vesicular body, thought to be the precursor of lamellar bodies. Microvillae are present on the surface of the cell.

Mono-oxygenase activity: *O*-de-ethylation of 3-cyano-7-ethoxycoumarin

Table 1 shows mono-oxygenase activity in Clara cells to be higher than those of Type II cells towards the *O*-de-ethylation of

Figure 4 Western blot of Clara and Type II cells probed for CYP2B1

Clara and Type II cells (5×10^4) isolated by fluorescence-activated flow cytometry as described in the legend to Figures 1 and 2 were subjected to SDS/PAGE on 9% (w/v) polyacrylamide gels, and the proteins were transferred electrophoretically to nitrocellulose sheets. CYP2B1 was revealed by reacting first with a rabbit-derived anti-CYP2B1 antibody and then with an alkaline-phosphatase-conjugated anti-rabbit antibody. Membranes were incubated with 5-chloro-4-bromo-3-indolyl phosphate substrate and Nitro Blue Tetrazolium as described in the Materials and methods section. Lanes 1–4 represent Clara cells and lanes 5–9 Type II cells. Lanes 1 and 5 are controls. The following rat pretreatments were used: lanes 2 and 7, Aroclor 1254; lanes 3 and 8, phenobarbitone; lanes 4 and 9, 3-methylcholanthrene. Lane 9 represents 1 pmol of CYP2B1 standard.

3-cyano-7-ethoxycoumarin. Activity was not significantly altered by freeze/thawing of the cells with the subsequent addition of NADPH to the reaction mixture (results not shown), suggesting that loss of this cofactor from intact cells was not rate limiting. Clara cells were more responsive to induction after pretreatment of rats with Aroclor or 3-methylcholanthrene, showing 10-fold higher activities than Type II cells. Aroclor was the most effective inducer with respect to this substrate in both Clara and Type II cells, showing 15- and 3.6-fold increases respectively, relative to controls. No activity could be detected in lung macrophages from control or pretreated rats.

CYP2B1 and CYP1A1 in Clara and Type II cells

Table 2 shows CYP2B1 to be 10-fold higher in Clara than in Type II cells, for control cells. Pretreatment of rats with phenobarbitone or Aroclor failed to cause induction of CYP2B1 in the unsorted and Type II cells and resulted in a decrease in its concentration in Clara cells (Figure 4). Although the polyclonal antibody used in the present experiments cross-reacted with CYP2B2, none could be detected in these lung cell preparations. There was a very low concentration of CYP1A1 in control cells, but after pretreatment of rats with Aroclor there was an approx. 2000-fold increase in the concentration of this cytochrome in Clara cells and a 280-fold induction in Type II cells. No CYP1A1 or 2B1 could be detected in lung macrophages from control or pretreated rats.

The concentration of cytochrome *P*-450 reductase protein in Clara and Type II cell preparations, determined by Western blotting, were 1.9 ± 0.5 - and 1.2 ± 0.2 -fold (mean $\pm$ S.E.M., $n = 4$) higher than in unsorted lung cells respectively. These values are relative, as the exact concentration of the protein standard was not known. Macrophages showed the presence of this enzyme to be 1.2 ± 0.2 -fold higher than in unsorted lung cells.

Table 3 Levels of GSH and GSSG in isolated sub-populations of rat lung

Single-cell dispersions of rat lung were prepared by subtilisin digestion. Cells from Aroclor-pretreated rats were incubated with diethoxyfluorescein, and Clara cells were isolated by flow cytometry on the basis of their fluorescence (DEF) or from control animals after treatment with monochlorobimane (MCB). Type II cells were isolated after incubation with Phosphine 3R, and macrophages on the basis of the forward-versus-side scatter. GSSG and GSH were estimated by h.p.l.c. after derivative formation with dansyl chloride as described in the Materials and methods section. Results represent means $\pm$ S.E.M. (4 experiments); nd, below the limit of detection (< 0.01 nmol/ 10^6 cells).

Cell type	Glutathione (nmol/ 10^6 cells)	
	GSH	GSSG
Unsorted	0.70 ± 0.05	0.06 ± 0.02
Clara (DEF)	2.87 ± 0.37	n.d.
Clara (MCB)	0.60 ± 0.13	0.14 ± 0.02
Clara (MCB) (+3h 0.2 mM <i>N</i> -acetylcysteine)	2.68 ± 0.28	0.13 ± 0.02
Type II	0.29 ± 0.05	n.d.
Macrophage	1.19 ± 0.13	0.02 ± 0.009

Figure 5 Resynthesis of GSH in Clara cells after reaction with monochlorobimane

Clara cells were isolated from rat lung after reaction with monochlorobimane as described in the legend to Figure 1. Clara cells (10^5) were incubated in supplemented Dulbecco's modified Eagle's medium containing *N*-acetylcysteine (0.2 mM) for the times indicated. Cells were recovered by centrifugation, and GSH levels were estimated after derivative formation with dansyl chloride (○). The intracellular GSH/monochlorobimane adduct (□) was estimated by h.p.l.c. as described in the Materials and methods section. Results represent means $\pm$ S.E.M. for 4 experiments.

GSH in isolated lung cells

The high level of blue fluorescence in the Clara-cell population, following treatment with monochlorobimane, suggested the presence of high intracellular levels of GSH. Estimation of the monochlorobimane/GSH adduct from these cells, by h.p.l.c., gave values of 1.71 ± 0.04 nmol/ 10^6 cells (mean $\pm$ S.E.M., $n = 4$). Monochlorobimane treatment did not totally deplete GSH in Clara cells. Estimates of the residual GSH by h.p.l.c., after derivative formation with dansyl chloride (Table 3), indicated the total GSH for Clara cells to be 2.4 nmol/ 10^6 cells. This was

very similar to the value estimated when Clara cells were isolated, on the basis of their mono-oxygenase activities, from Aroclor-pretreated rats (Table 3). GSH levels in Clara cells were about 9 times higher than in Type II cells. Figure 5 shows that GSH depleted from Clara cells by monochlorobimane treatment was resynthesized to 2.7 nmol/ 10^6 cells over 3–4 h when incubated in the presence of *N*-acetylcysteine (0.2 mM). This was accompanied by the loss of the GSH/monochlorobimane adduct from the cells.

γ -glutamyltranspeptidase activity in isolated cells

γ -Glutamyltranspeptidase activity of Clara cells (58 ± 6 nmol/min per 10^6 cells; $n = 4$) was 6-fold higher than in Type II cells (8.5 ± 2.1 nmol/min per 10^6 cells; $n = 5$). Lower activity was observed in macrophages and in unsorted cells (1.1 ± 0.5 and 5.1 ± 0.9 nmol/min per 10^6 cells respectively; $n = 5$).

DISCUSSION

The occurrence of Clara cells in rat lung is low ($< 2\%$) [8], and different proteolytic enzymes can yield different proportions of pulmonary cell types [21]. An aim of the present work was to obtain the cell types of interest in high numbers as very pure sub-populations with good biochemical and morphological integrity. Although subtilisin digestion of rat lung gave a yield of single cells that was 30–50% lower than previously reported using trypsin or protease VII [22,23], Clara cells were isolated by subtilisin as a selectively higher proportion (2.8%) of the total unsorted population than by either trypsin or protease XIV (J. Martin, unpublished work). The use of proteases for cell isolation may damage cells, and the results from light microscopy in this study show the presence of vacuoles in a number of Clara cells after isolation (Figure 2a). However, high-power transmission electron micrographs (Figures 3a and 3b) of Clara and Type II cells from rat lung show membranes and subcellular structures to be intact, and the major characteristic features to be well defined. Previous studies have also indicated that Clara or Type II cells do not suffer from major damage when isolated from rabbit lung by using such proteolytic enzymes [21].

Monochlorobimane has been used as a fluorescent probe for investigating the heterogeneity of GSH in cells in culture [24]. We have shown that this technique can be used to label and isolate Clara cells of $> 95\%$ purity from rat lung. Assuming all the different types of unsorted lung cells contain GSH *S*-transferases, necessary to conjugate GSH with monochlorobimane [25], analysis by fluorescence-activated flow cytometry suggests that Clara cells have the highest GSH concentrations (Figure 1). Monochlorobimane treatment depleted the intracellular GSH of the Clara cells (Table 3), but sufficient was retained to avoid the increased lipid peroxidation and cell death associated with rapid GSH depletion as has been described in isolated hepatocytes [26]. Clara cells had a high viability, as judged by Trypan Blue exclusion, and GSH levels could be restored by incubating cells in the presence of *N*-acetylcysteine (Table 3). This level of GSH in both unsorted cells and sorted Clara cells as determined by h.p.l.c. was 4.5-fold higher than previous estimations [9,23]. This may reflect the short perfusion time and the low concentration of dispersal enzyme. In Clara cells in particular, previous purification techniques using density-gradient centrifugation and centrifugal elutriation probably led to loss of GSH which was not replenished. Depletion of GSH of Clara cells by reaction with monochlorobimane and its replenishment by *N*-acetylcysteine resulted in a level similar to that in Clara cells determined immediately after sorting by dimethoxyfluorescein.

This would suggest the cells' GSH level was maintained during the enzyme dispersal and physical passage through the flow cytometer.

γ -Glutamyltranspeptidase activities were 50-fold higher in Clara cells than in macrophages, and 6-fold higher in Type II cells. Although the precise role of this enzyme in such cells is not clear, high activities in Clara cells may be associated with high intracellular GSH levels. It has been suggested that Clara and Type II cells may import GSH from the extracellular fluid lining the lower airways, where it is present in high concentration [11], since immunohistochemical localization of this enzyme shows it to be present on the apical surfaces of such cells [10].

Despite a degree of vacuolation, mono-oxygenase activities could be demonstrated in Clara cells (Table 1). Such activities were not enhanced after freeze-thawing and the addition of exogenous NADPH. Although the concentration of NADPH was not determined, results suggest it had not been depleted from the cells to an extent where it was rate-limiting. Other components, such as cytochrome *P*-450 reductase, must also have been functionally present. Studies of mono-oxygenase activity and CYP isoenzymic forms in individual cell types of rat lung have previously been very limited. We have shown mono-oxygenase activities towards 3-cyano-7-ethoxycoumarin to be about 2-fold higher in Clara than in Type II cells (Table 1). This substrate was chosen since it allows continuous monitoring of reaction rates, and the assay is more sensitive than that using ethoxyresorufin and is not subject to interference by DT-diaphorase [16]. The 3-fold induction of this activity with 3-methylcholanthrene was similar to that reported with 7-ethoxycoumarin as the substrate and β -naphthoflavone as the inducing agent [8]. In contrast with previous studies [3,9] pretreatment of rats with 3-methylcholanthrene resulted in little induction of mono-oxygenase activities in Type II cells (Table 1). However, pretreatment with 3-methylcholanthrene did result in a substantial (300-fold) induction of CYP1A1 protein (Table 2). The reason for this apparent discrepancy is not known; the possibility that phosphine 3R or its metabolites might have acted as an inhibitor of mono-oxygenase activities was not investigated.

The present results show that pretreatment of rats with Aroclor 1254 results in 3–4-fold higher levels of induction of CYP1A1 and mono-oxygenase activity that does 3-methylcholanthrene pretreatment (Tables 1 and 2). They also suggest an explanation of why *O*-de-ethylation of diethoxyfluorescein to fluorescein, previously used to label and isolate hepatocytes from control rats [27], was only successful in labelling Clara cells from the lungs of Aroclor 1254-pretreated and not control animals. Clara cells isolated by this independent criterion proved useful in the determination of uncompromised levels of intracellular GSH (Table 3).

Although CYP2B1 is the major isoenzyme in control-rat lung, the presence of additional isoenzymes, e.g. CYP4B1 and CYP2E1, has been described [28,29]. Immuno-histochemical evidence suggests that CYP isoenzymes may also be present in cell types apart from Type II and Clara cells, e.g. endothelial and ciliated epithelial cells [6,7]. The contribution of the three different cell types' complement of CYP isoenzymes to the overall response of the lungs to xenobiotics has yet to be fully assessed.

In conclusion, small numbers of highly purified subpopulations, derived by the flow-cytometric method described here, have been successfully used to characterize enzyme profiles of the different cell types. However, this technique also offers the possibility of studying the effect of lung toxins on nuclear events. For example, normal and abnormal gene expression following toxic insult could be assessed by use of the polymerase chain reaction technique.

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