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Type II alveolar epithelial cell *in vitro* culture in aerobiosis

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ABSTRACT: A method of Type II alveolar epithelial cell culture in aerobiosis has been developed. Isolation of Type II cells was performed by digesting guinea-pig lung tissue with crude trypsin and elastase and using discontinuous Percoll density gradients. The Type II cells, as identified by light and electron microscopy, were cultured in aerobiosis for up to six days, in direct contact with the atmosphere in conditions mimicking those present in the lower respiratory tract. Significant activities of cellular superoxide dismutase (SOD), manganese dependant superoxide dismutase (Mn-SOD), catalase and glutathione peroxidase (GSH-Px) were found at the time of isolation. In contrast, cell glutathione content varied widely from one experiment to another. Changes of antioxidant enzymes were evaluated during cell culture in aerobiosis. SOD, Mn-SOD and catalase were significantly decreased after three days but were not significantly different between a three day and six day culture. Antioxidant changes did not influence the cell culture. In marked contrast, decrease in cell glutathione was associated with rapid cell death, whereas good cell survival was obtained at high levels of cell glutathione. Cell culture in aerobiosis will permit a precise evaluation of the effects of gases, particularly oxidant gases, on a primary culture of Type II alveolar epithelial cells.

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Type II alveolar epithelial cells, also called granular pneumocytes, are the cuboidal cells which form part of the alveolar wall. There is evidence that Type II cells synthesize and secrete pulmonary surface-active material [1], maintain the alveolar epithelium by serving as stem cells for both Type I and Type II cells and, probably, transport fluid from the alveolar subphase into the interstitium [2, 3].

Investigating the properties of pure populations of lung cells may be useful for precise elucidation of specific functions. Thus, during the last ten years a number of methods have been developed for obtaining Type II cells from mammalian lungs [4–10]. In addition, Type II cells in primary culture have been used to study processes involved both in the genesis of chronic lung diseases and in the toxic effects of exogenous agents, in particular oxidants [4, 5, 11–15]. However, until now, these *in vitro* models have been performed using classical monolayer cultures with cells adhering to the flask and covered with nutrient medium and have not permitted evaluation of the cytotoxic effects of gases, especially the direct effects of low concentrations of oxidant gases or atmospheric pollutants on lung cells.

With this in mind, we developed an *in vitro* model of Type II cell culture in aerobiosis adapted from an original model of alveolar macrophage culture in the gas phase previously described in our laboratory [16]. The aims of this work were to isolate Type II cells, to cultivate them in aerobiosis and to evaluate the antioxidant defences of these cells and their changes during cell culture. In the present study, the cells were obtained as a primary isolation from guinea-pig lungs and cultured for up to six days.

Material and methods

Cell isolation

Type II alveolar epithelial cells were obtained from male and female, albino Dunkin Hartley guinea-pigs weighing 400–500 g. The method of isolating Type II cells was adapted from that described by KIKKAWA and YONEDA [4] and MASON *et al.* [5] for isolating Type II granular pneumocytes from the rat lung. One hundred experiments were performed, each of them comprising six animals. Guinea-pigs were anaesthetized by intraperitoneal injection of 100 mg of sodium

pentobarbital. The chest was opened and the carotids were sectioned. Physiologic saline solution with ethylene diamine tetra-acetic acid (EDTA) 3mM was injected at maximal manual pressure via the inferior vena cava to wash the vasculature until the liquid flowing away via the carotids was clear. Manual ventilation of the lungs through a tracheal cannula was carried out simultaneously. The lungs were removed from the thorax and transferred to a petri dish.

The first lung lavage was performed with Hank's solution buffered with hydroxyethylpiperazine ethanesulphonic acid (HEPES) 18 mM without calcium and magnesium, and with gentamycin (50 $\mu g \cdot ml^{-1}$) and amphotericin B, to eliminate part of the free lung cells. An emulsion of fluorocarbon and albumin (0.5 ml of fluorocarbon diluted with 4.5 ml of 1% albumin Hank's HEPES, pH 7.4) was instilled through the tracheal tube to facilitate the removal of macrophages by increasing their density. The lungs were placed, at 37°C, in a bottle containing a physiological solution with antibiotics and fungicides, in order to allow remaining macrophages to ingest the heavy emulsion. After 15 min, the lungs were lavaged ten times with 50 ml Hank's HEPES to remove additional macrophages as well as fluorocarbon. The enzymatic digestion of the lungs was carried out by instillation of 5 ml Hank's solution buffered with HEPES (pH 7.4) containing crude trypsin 1.5 $mg \cdot ml^{-1}$ (Sigma Chemical Co., St Louis, MO), elastase 1.5 $U \cdot ml^{-1}$ (Sigma) and deoxyribonuclease (DNAase) 30 $\mu g \cdot ml^{-1}$ (Sigma). The lungs were immersed in saline solution at 37°C.

After a 10 min contact, a 5 ml solution of trypsin and elastase was again instilled; the lungs were immersed in saline solution at 37°C for 10 min, transferred to a petri dish and filled with a solution of soya bean trypsin inhibitor (Sigma) in Hank's HEPES (pH 7.4) solution containing DNAase 30 $\mu g \cdot ml^{-1}$ and foetal calf serum 10% to stop the action of trypsin and elastase. Since Type II cells are susceptible to oxidative injury, we also investigated whether exogenous glutathione (GSH)) can provide protection in Type II cells against the oxidative injury during isolation and culture in aerobiosis. To accomplish this, GSH 20 μM was added to the solution in ten experiments.

The peripheral lung tissue was removed from the bronchial tree with sharp scissors and the tissue fragments were sectioned into small pieces. The suspension of tissue was shaken vigorously for 2 min and filtered through gauze and nylon (NY 25 HC) to eliminate the Type I pneumocytes. The separation of Type II cells was obtained by velocity sedimentation in a unit gravity cell separator [17]. With the sedimentation chamber in a vertical position, a discontinuous density gradient of Percoll (Pharmacia AB, Uppsala, Sweden) was made as follows:

Density 1.100 (100 ml)
1.085 (30 ml)
1.050 (40 ml)
1.035 (30 ml)
1.010 (20 ml)

A 50 ml parenchymal cell suspension and 50 ml Hank's HEPES were prepared and were layered over the gradient so as to obtain a total quantity of 320 ml. After closure of the sedimentation chamber, the chamber was reorientated from the vertical to the horizontal position at speed 2. By this procedure the surface area increased and the thickness of layers reduced by a factor of 12.5. After 30 min in the horizontal position, the sedimentation chamber was returned at lowest speed to its vertical position. After a further 15 min, the 32 fractions were removed from the cell separator in 10 ml fractions with a specially designed siphoning device.

One hundred and forty ml (fractions 1–14) were removed and five fractions of ten ml (fractions 15–19) were recovered (fig. 1). Each 10 ml fraction was observed by phase contrast microscopy to eliminate the fraction containing polymorphonuclear neutrophils and eosinophils (usually fraction 19, table 1). The harvested cells were centrifuged at low speed (5 min, 500 g) and recovered in Eagle's basal medium (BME) (Gibco Laboratories, Grand Island, NY) without serum. This cellular suspension was placed in a glass bottle for 15 min. The alveolar macrophages were rapidly fixed. The non-adherent cells were washed three times in BME with added foetal calf serum 10%. The remaining thirteen fractions (20–32) were then removed and examined.

Table 1. – Distribution of cell types according to the fractions of density gradient

10 ml fractions	Bronchial cells and unidentified lung cells %	Alveolar macrophages %	Neutrophils %	Eosinophils %	Type II cells %	Lymphocytes %	Monocytes Plasmocytes %
6–10	73.5±23.5		0.50±0.5	0.50±0.5		21.3±22	4.25±4
11–12	60.8±12		0.75±1	0.50±0.5		38.0±13	
13–14	23.3±12		1.00±1.5	4.00±6.6	15.0±13	51.0±16.8	5.80±2
15	1.8±2.3	2.5±3.8	4.00±4.5	2.25±1.5	73.0±4	16.5±7.6	
16–17	0.5±1	5.5±3.3	3.80±1.7	7.80±2.2	80.8±4.6	1.8±2.3	
18		5.0±3	12.00±2.2	8.00±2	73.0±2.6	2.0±1.5	
19		6.8±7.8	43.50±29	30.00±37	17.5±5.7	2.2±3.8	
20–22		16.5±13.7	27.80±3.1	55.80±45			

Fractions 1–5 contained cell fragments. Fractions 23–32 contained most macrophages and polymorphonuclear cells with fluorocarbon. Results are expressed as mean±SD.

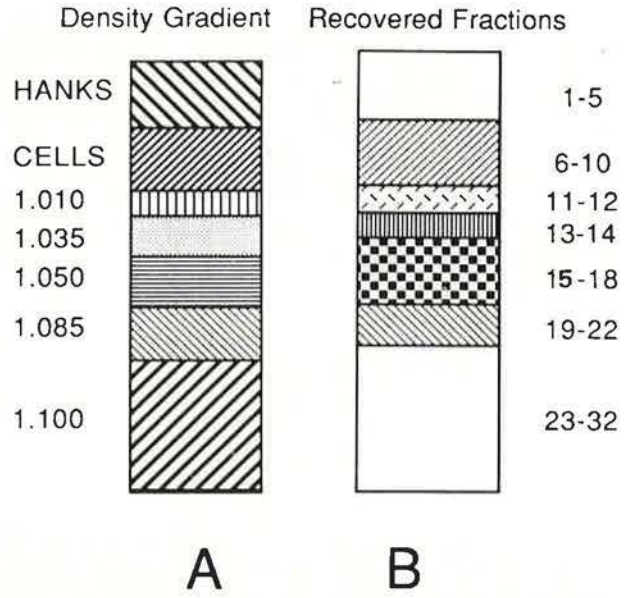


Fig. 1. – A schematic representation of the sedimentation chamber in vertical position: A) solutions of the different densities, cellular suspension and Hank's HEPES solution; B) different fractions obtained after sedimentation.

Cell identification

Identification of the cells was carried out by light and electron microscopy.

For light microscopy air-dried unfixed smears of the cell isolates were stained by the Papanicolaou method without acid alcohol. The inclusions of Type II cells were easily identified as deep blue granules, each surrounded by a clear halo.

For electron microscopy the cells in pellets were fixed in 1% glutaraldehyde in cacodylate buffer 0.1 M, pH 7.2. After 12 h at 4°C the cells were washed in cacodylate buffer and postfixed in osmic acid for one hour. The specimens were dehydrated with acetone then fixed in epoxyaraldite. The cells were stained with lead citrate according to Reynold's modified method and examined under a Hitachi type HV 12 electron microscope.

Culture of Type II pneumocytes in aerobiosis

Cell numbers were determined manually with a standard haemocytometer. The cell viability was judged by the trypan blue dye exclusion test. The cells were suspended in BME with glutamine (2 mM) and foetal calf serum 10% in order to obtain $10 \cdot 10^6$ cells·ml^{-1}.

The cell cultures were performed according to the method previously described for alveolar macrophages [16]. The Type II pneumocytes were layered on a triacetate cellulose membrane (Gelman Sciences, inc., Ann. Arbor, Michigan) of 0.2 µm porosity. This membrane was applied to the surface of a reservoir filled with nutrient medium in order to be saturated by capillary action. The cells were in direct contact with air without any interposition of liquid medium. Cell preparations were placed in special chambers (IGR caisson, Lequeux S.A., Paris) in purified reconstituted air (Alpha gaz S.A., France), saturated with water (37°C) and enriched with CO_2 5%. With this experimental model, Type II cells were cultured in aerobic conditions mimicking those existing in the lower respiratory tract to permit evaluation of the biological effects of oxidant gases on cell behaviour with special attention to the changes of the antioxidant equipment. Thus, in order to improve the cell culture conditions, Type II cells were cultured in aerobiosis either with pure nutrient medium or with modified nutrient medium as follows:

1) nutrient medium was BME with Earle's salts and glutamine (2 mM) (Gibco) enriched with foetal calf serum 10%, antibiotics and amphotericin B;

2) in some experiments, embryonic extracts obtained from foetal guinea-pig lungs (45th gestation day) were added to the nutrient medium. The embryonic lungs were sectioned into fine pieces with sharp scissors in a 1 ml Hank's HEPES solution (pH 7.4). The suspension was shaken vigorously for 1 min and centrifuged for 10 min at 800 g. The surpernatant called "lung extract" was added to the nutrient medium, 1 ml in 50 ml; and 3) in other experiments three small pieces of embryonic lung called "lung explants" were introduced into a reservoir filled with nutrient medium.

Biochemical analyses

Two types of cellular antioxidant activity were studied: 1) antioxidant enzymes, that is superoxide dismutase (SOD), manganese dependent SOD (Mn-SOD), catalase, glutathione peroxidase (GSH-Px) and 2) glutathione. In an additional study, the antioxidant enzymes were compared between Type II cells and alveolar macrophages obtained from the same animals [16].

1) Superoxide dismutase (SOD) assay. The Type II pneumocytes were washed and centrifuged three times at 500 g in Hanks HEPES. They were then resuspended in phosphate buffer pH 7.8 at the rate of $6 \cdot 10^6$ cells in 2 ml. The cellular suspension was sonicated on ice (MSE sonifier at a 100 W power to 20 kHz for 30 s). After centrifugation at 800 g for 10 min at 4°C the supernatants were removed and treated with Triton X 0.2% v/v. After 30 min incubation the solutions were centrifuged at 45,000 g for 15 min at 4°C and the supernatants were assayed for SOD using the method of McCord and Fridovich [18] modified by Crapo and McCord [19] with respect to pH (pH 10). This method is based on the capacity of SOD to inhibit the cytochrome C reduction mediated by O_2^- generated during the oxidation of xanthine catalysed by xanthine oxidase. The manganese dependent SOD (Mn-SOD) activity was measured by adding KCN 10^{-3}M. The unit of enzymatic activity was defined by the 50% inhibition of reduction of cytochrome C in a final volume of 3 ml.

2) Catalase assay. Cellular extraction was performed using the same method as was used for SOD assay but

the cells were resuspended in phosphate buffer pH 7 ($2 \cdot 10^6$ cells·ml^{-1}). Catalase was measured using the method of BEERS and SIZER [20] involving reduction of H_2O_2 per min at 25°C; 0.1 ml of supernatant was added to 2.9 ml of the substrate solution (H_2O_2); for reproducible results the A_{240} should be between 0.55 and 0.52. The time required for A_{240} to decrease from 0.45 and 0.40 was noted and corresponded to the decomposition of 3.45 µM of H_2O_2 in the 3 ml solution.

3) Glutathione peroxidase (GSH-Px) assay. The GSH-Px activity was assayed according to the method of PAGLIA and VALENTINE [21] modified by HOLMES *et al.* [22]. Cellular extraction was the same method as that used for the catalase assay except for the protein concentration which was more important ($10 \cdot 10^6$ in 2 ml). The reaction was initiated by the addition of 0.1 ml of 2.2 mM H_2O_2 to the reaction mixture. The reaction mixture contained 2.46 ml of 0.05 M phosphate buffer pH 7 containing 5 mM EDTA, 0.1 ml of 8.4 mM reduced nicotinamide-adenine-dinucleotide phosphate (NADPH), 0.1 ml of 0.15 M reduced glutathione, 20 µl of 0.562 M azide, 20 µl of (4.6 U) glutathione reductase (Sigma Chemical Co) and 200 µl of cellular extract. The change in the optical density was read at 340 nM between 2' and 4'. In the absence of enzyme protein the change was always less than 5% of the total reaction. The data were expressed as nM NADPH oxidized to NADP by using the extinction coefficient of $6.2 \cdot 10^3 \cdot M^{-1} \cdot cm^{-1}$ at 340 nM.

4) Intracellular glutathione assay. $1.4 \cdot 10^6$ Type II pneumocytes were placed in 500 µl of 0.05% w/v Triton X 100. Samples of the lysates (380 µl) were acidified with 20 µl of 0.1 N HCl. The protein was precipitated by the addition of 20 µl of 50% w/v sulphosalicylic acid and removed by centrifugation [23].

The supernatants were assayed for total glutathione (GSH and GSSG) by an enzymatic recycling procedure: glutathione was sequentially oxidized by 5-5' dithiobis 2-nitrobenzoic acid (DTNB) and was reduced by NADPH in the presence of glutathione reductase at 30°C [24]. 2 nitro 5 thiobenzoic acid formation was monitored at 412 nM between 10' and 16' by comparison of the result with the standard curve. GSH standards contained Triton X 100, sulphosalicylic acid and HCl in quantities identical to the samples.

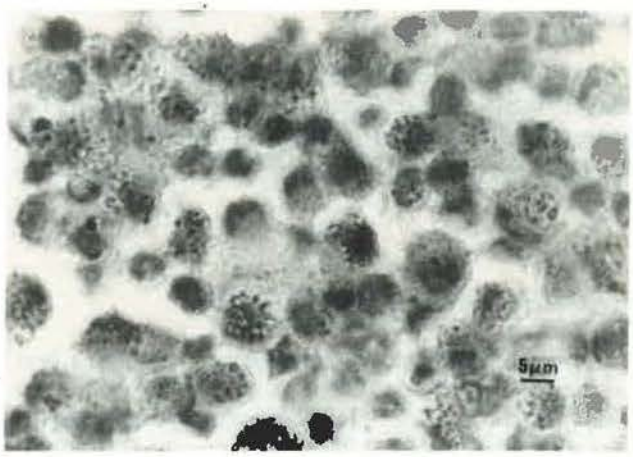


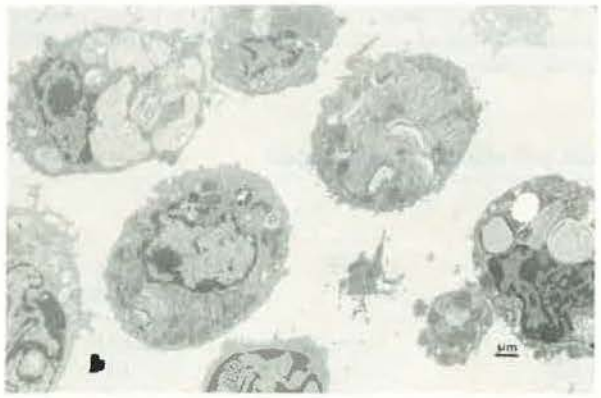
Fig. 2. – A smear of isolated cells from pooled fractions 15–18 stained by the Papanicolaou modified method. The inclusions of the Type II cells were easily identified as deep granules each surrounded by a clear halo (x1200).

Protein assay

Protein content of cells was measured by the method of Lowry. Activities were expressed as specific activity per 10^6 cells and/or by mg of protein.

Evaluation of cell viability during culture

The cell viability was estimated by measuring ATP content according to the method of MCELROY and SELIGER [25] modified by VOISIN *et al.* [16].



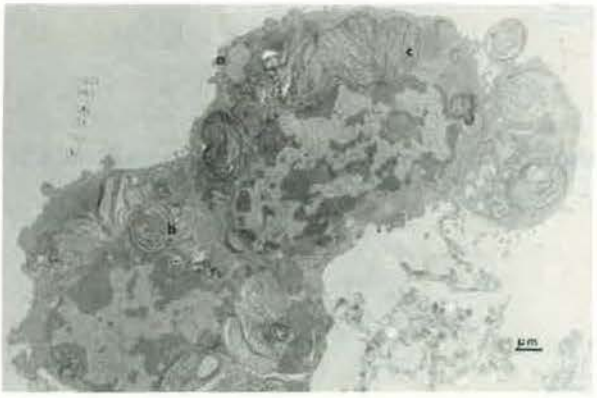


Fig. 3. – Electron micrograph of isolated cells: A) The Type II cells are identified by the presence of lamellar bodies (x6000); B) A group of three cells with different stages of lamellar body maturation from small lipid small drops (a), to multicentric (b) and parallel (c) lamellar bodies (x7000).

Statistical analysis

All results were expressed as mean±SD. All comparisons of statistical significance were carried out using the non-parametric test of Mann and Whitney and the Wilcoxon test. $p<0.05$ was considered as significant.

Results

Identification of cell types in suspension

In four experiments identification of cell types was performed before separation on density gradient. The total number of cells obtained from five guinea-pigs was $216\pm48\times10^6$ cells. After separation on a discontinuous gradient, the percentage of recovered cells was 66±10%. The remainder of the cells might have been lost in the separation procedure due to clumping or due to adherence to the walls of the separator chamber. Of the recovered cells, 26±8% were located in fractions 1–14, 31±5% in fractions 15–19 and 9±4% in fractions 19–32 (fig. 1).

Table 1 shows that most of the Type II cells were found in fractions 15–18. The major contaminants in fraction 15 were small lymphocytes and in fractions 18 and 19 polymorphonuclear cells. As expected alveolar macrophages were present mostly in fractions 20–32.

After adherence on glass (fractions 15–18) the number of cells decreased by 12±5% and the purity of Type II pneumocytes increased to 80–90%.

Table 2. – Antioxidant activities of Type II cells

	n	$U\cdot10^6$ cells	$U\cdot mg^{-1}$ protein
Total SOD	40	1.78±0.41	10.83±2.4
Mn-SOD	28	0.45±0.13	2.75±0.75
Catalase	27	37.50±11.5	201.00±58
GSH-Px	9	1.45±0.85	10.76±5

SOD: superoxide dismutase; Mn-SOD: manganese-dependent SOD; GSH-Px: glutathione peroxidase. Results are expressed as mean±SD.

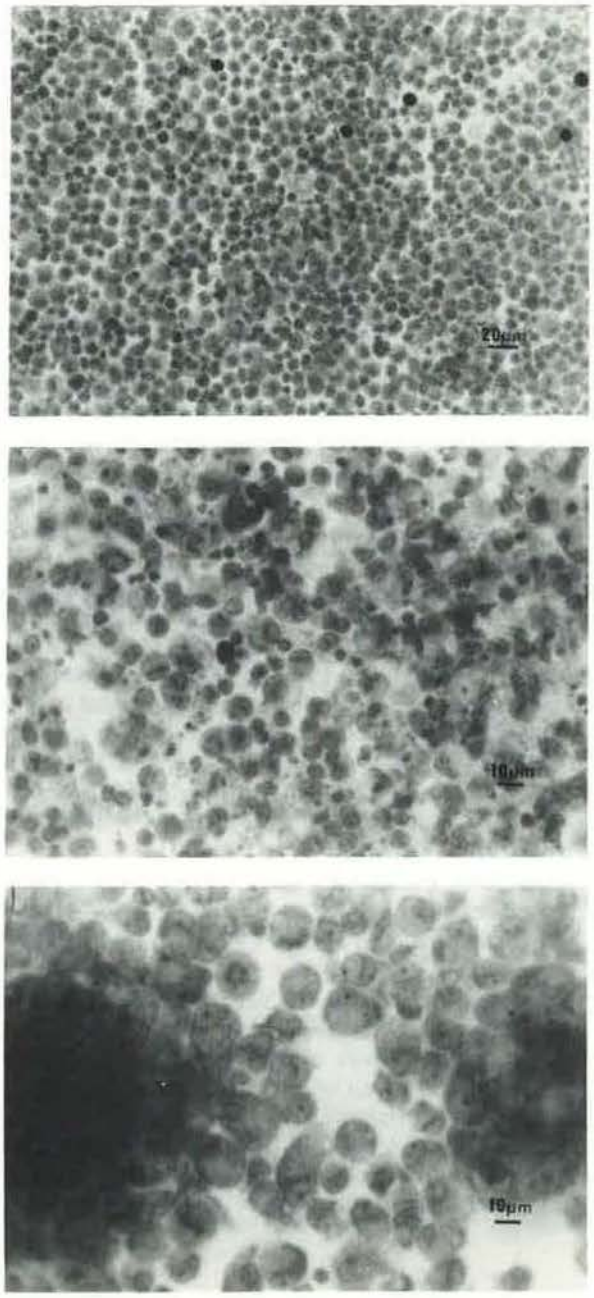


Fig. 4. – Type II cell culture in aerobiosis. The Type II cells stained by May-Grunwald method. A) 0 h culture (x300); B) 24 h culture (x500); C) six day culture (x500).

Table 3. – Comparison of antioxidant enzyme content of Type II cells and alveolar macrophages

		$U\cdot10^6$ cells		$U\cdot mg^{-1}$ protein	
	n	Type II cells	Alveolar macrophages	Type II cells	Alveolar macrophages
Total SOD	16	1.8±0.37*	1.5±0.33	11.0±1.9*	8.4±2.9
Catalase	7	41.2±11.1*	48.8±16.2	223.0±60*	256.0±67
GSH-Px	6	1.4±1.1	1.8±1.1	9.4±6.6	11.4±5.4

Results are expressed as mean±SD. * significantly different from alveolar macrophages, $p<0.05$ Wilcoxon test.

Characteristics of Type II cells at isolation

A mean of $8.4\pm2.2\times10^6$ guinea-pig cells was obtained from the density gradient with a purity of Type II cells ranging from 80–90%. The Type II cell fraction obtained showed 94±4% trypan blue dye exclusion.

Morphological study. Stained smears of harvested cells showed the inclusions of Type II cells as deep blue granules each surrounded by a clear halo (fig. 2).

In phase contrast microscopy the cells were round, well individualized with intracytoplasmic granules. In these preparations some groups of two or three cells, so-called clusters, were observed. When Type II cells were examined by transmission electron microscopy, they were found to be intact with well-defined lamellar inclusion bodies within the cytoplasm and microvilli at the cell border (fig. 3). The mitochondrial matrix was electron-dense in most of the Type II cells. On the whole, the appearance of the Type II cells was similar to that observed in the lung tissue fixed immediately after death.

Antioxidant activities. Results of antioxidant enzymes are summarized in table 2. Significant activites of SOD, Mn-SOD, catalase and GSH-Px were found in Type II cells. Comparison between Type II cells and alveolar macrophages demonstrated that SOD activity was higher in Type II cells whereas catalase activity was lower. GSH-Px content was not significantly different between Type II cells and alveolar macrophages (table 3). Strikingly, glutathione cell content varied widely from one experiment to another (902 ± 540 ng·10^6 cells; 4839 ± 2365 ng·mg^{-1} protein, n=18). The addition of GSH to the solution during the isolation phase was not associated with a significant increase in glutathione content (768 ± 508 ng·10^6 cells; 3767 ± 2580 ng·mg^{-1} protein, n=10).

Type II cell culture in aerobiosis

Morphologic study. A May-Grunwald Giemsa stained cell preparation on membrane is shown in figure 4A. A homogeneous repartition of readily identifiable Type II cells is demonstrated. After a 24 h culture (fig. 4B), Type II cells grouped in twos or threes and at 48 h began to form domes which were seen to be numerous with visible mitoses in a 6 day culture (fig. 4C).

When examined by transmission electron microscopy, after 24 h (fig. 5) or 48 h (fig. 6) of culture in aerobiosis, Type II cells displayed morphological characteristics similar to Type II cells examined immediately after isolation. When cultured in the presence of lung extracts or lung explants, lamellar bodies could still be observed in a 6 day culture (fig. 7).

Evaluation of cell culture viability. Mean ATP cell content did not decrease significantly after 1, 2, 3 or 6 days (853 ± 198, 1054 ± 337, 1000 ± 319 and 1235 ± 647 ng/membrane, respectively ($1\cdot10^6$ cells) suggesting persistent metabolic activity of Type II cell culture in aerobiosis. However, death of cell culture was observed in the presence of a high percentage of polymorphonuclear cells ($14.9\pm7\%$) whereas a good cell survival in culture was associated with a significantly lower percentage of polymorphonuclear cells at the isolation time ($7.1\pm6\%$, $p<0.01$).

Sequential evaluation of antioxidant activities during gas phase culture

Total SOD content was significantly ($p<0.001$) lower after a 3 day culture than at isolation but did not change after a 6 day culture (fig. 8). Addition of lung

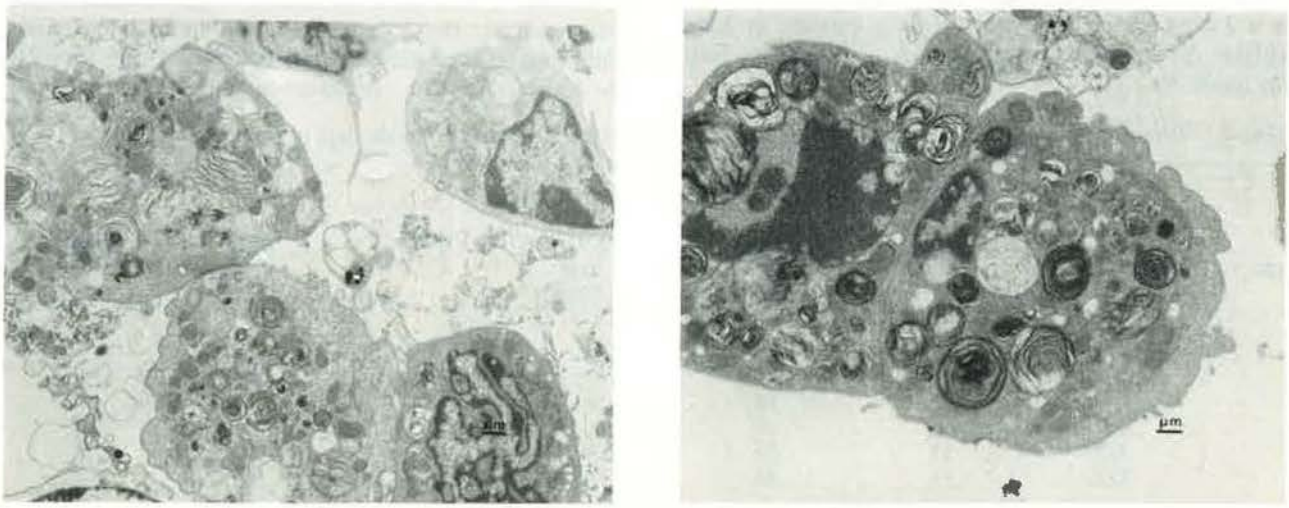


Fig. 5. – Transmission electron micrograph of several Type II cells after a 24 h culture in aerobiosis (x6000).

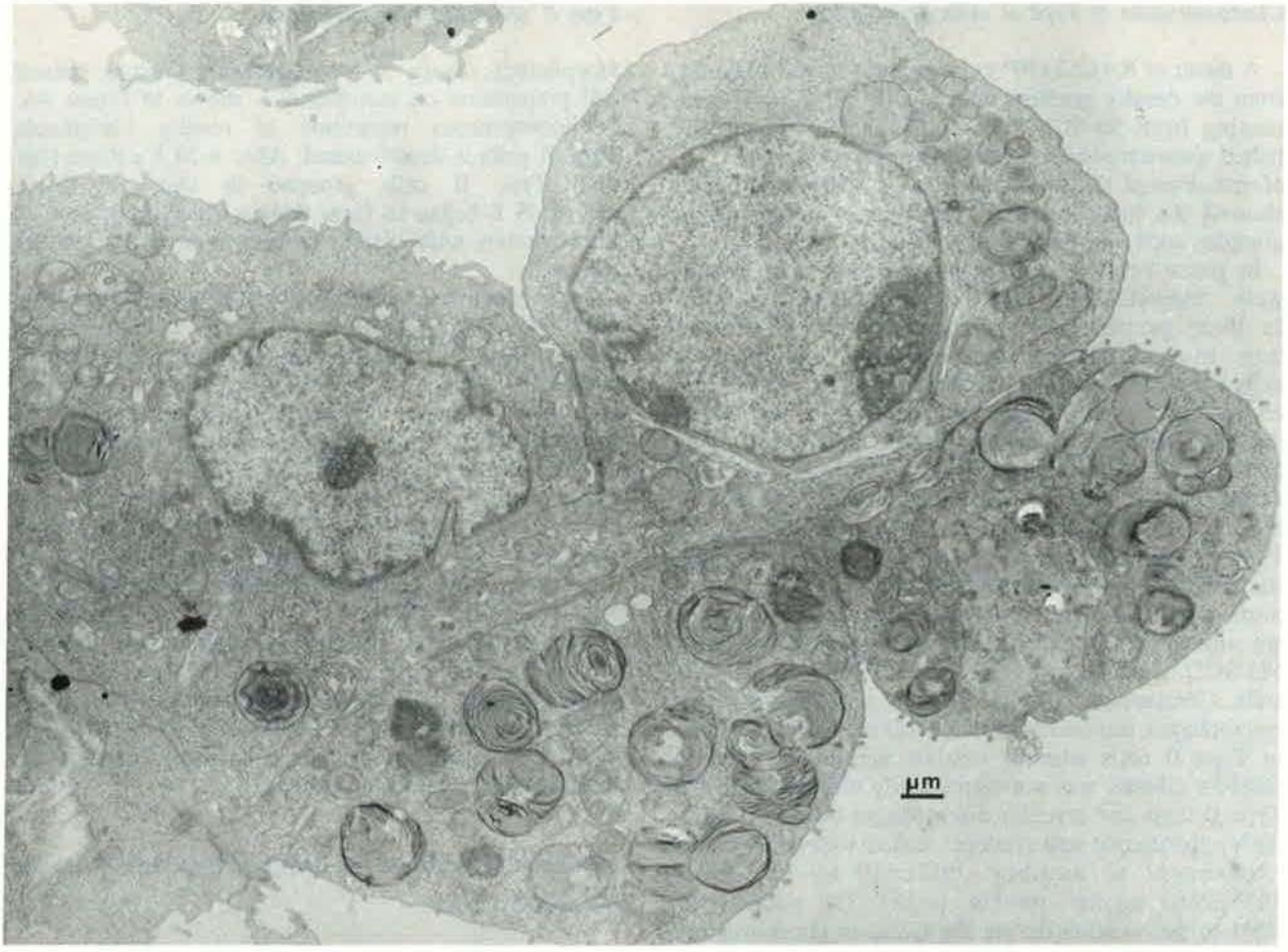


Fig. 6. – Transmission electron micrograph of several Type II cells after a 48 h culture in aerobiosis (x6000).

extracts and/or lung explants to the culture medium were associated with a significant increase in total SOD after a 3 day culture ($p<0.01$) and after a 6 day culture ($p<0.001$). However, as shown in figure 8, changes in total SOD were largely due to changes in Mn-SOD. Catalase activity significantly decreased after a 3 day culture. Addition of lung extracts or lung explants did not significantly increase cell catalase content (table 4).

Sequential assay of cell glutathione and of cell ATP content is shown in figure 9. A strong correlation was found between cell ATP content and cell gluathione at a 3 day culture ($r=0.79$, $n=18$, $p<0.001$). High values of cell glutathione after 3 or 6 days were associated with a good survival of Type II cells in gas phase culture. These data suggest an important relationship between cell glutathione content and cell survival in aerobiosis. The presence of lung extracts or lung

Table 4. – Changes in catalase activity during cell culture in aerobiosis, according to various nutrient media

Expt	Day 0	Day 3			Day 6		
		BME	BME + lung extracts	BME + lung explants	BME	BME + lung extracts	BME + lung explants
1	160	0	0	0	0	0	0
2	196	33	43	ND	80	129	ND
3	201	39	44	37	ND	ND	ND
4	207	6	0	ND	23	24	ND
5	235	37	34	31	ND	ND	65
6	321	31	34	26	153	96	126

Results are expressed as $U \cdot mg^{-1}$ protein; BME: Eagle's basal medium; ND: not done.

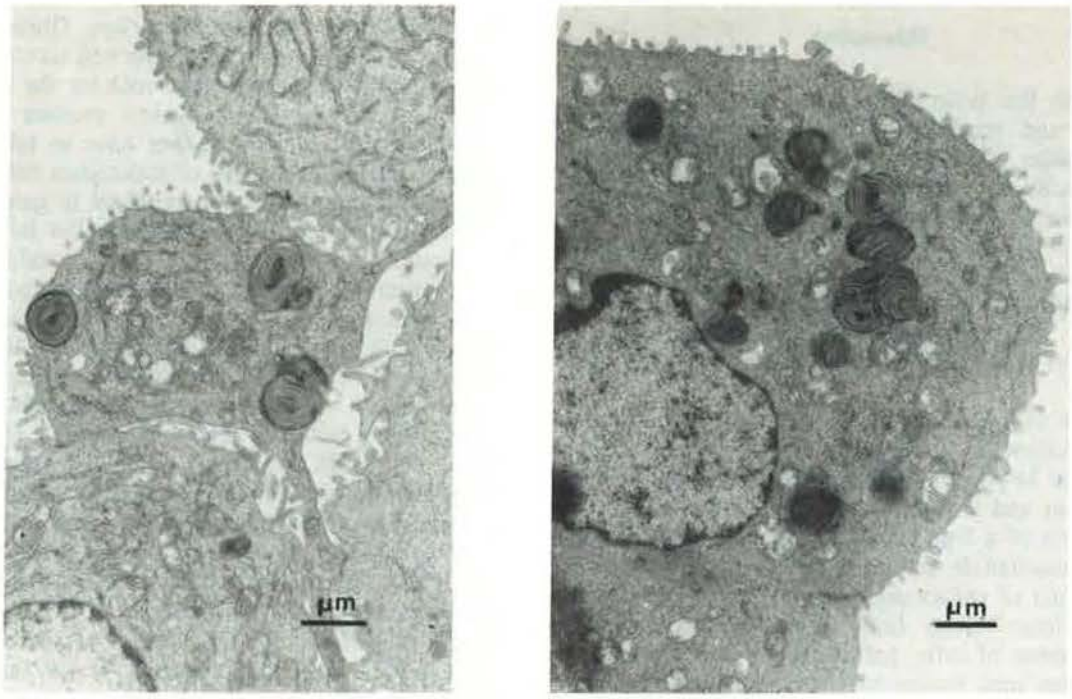


Fig. 7. – Transmission electron micrograph of cells maintained in primary culture at six day culture (with lung explants in nutrient medium) (x8400).

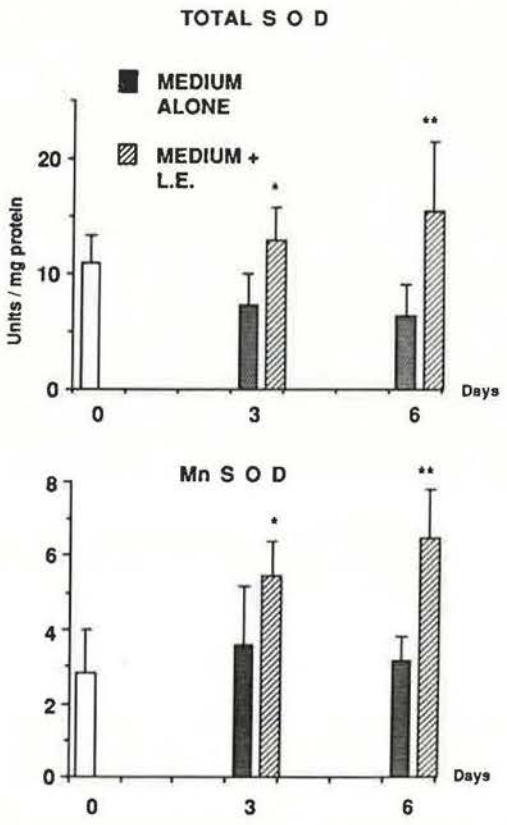


Fig. 8. – Evaluation of cellular SOD activities for a six day gas phase culture in nutrient medium alone and nutrient medium enriched with lung extracts or lung explants (L.E.). Upper panel: total SOD content; lower panel: nonsensitive SOD KCN 10^{-3} M content (Mn-SOD). *: $p<0.01$; **: $p<0.001$.

explants in the nutrient medium did not significantly increase cell glutathione content after 3 day culture (5807±5164 ng·mg^{-1} and 4287±3742ng·mg^{-1} protein, respectively, n=6) compared to values observed when Type II cells were cultured with BME (4741±2725 ng·mg^{-1} protein, n=6). However, after a 3 day culture the cell glutathione content was significantly higher when cell isolation was performed with GSH (9915±6345 ng·mg^{-1} protein, n=9, $p<0.01$) than when cell isolation was performed without GSH (3717±3084U·mg^{-1} protein, n=15).

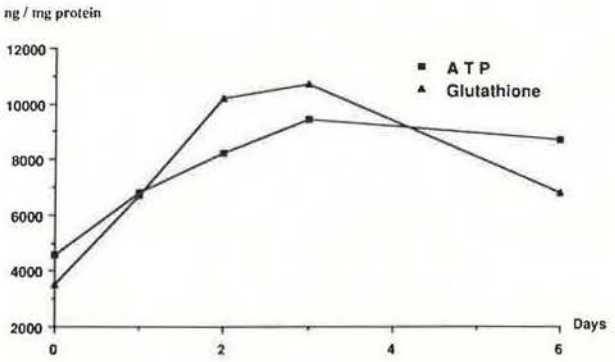


Fig. 9. – Sequential assay of glutathione and ATP content of Type II cells during cell culture in aerobiosis.

Discussion

Although the main functions of Type II cells are synthesis and secretion of pulmonary surface-active material, there is evidence that a second major function of these cells is to regenerate a continuous epithelium after diffuse alveolar injury, in particular after oxidative injury by oxidant gases, drugs or various toxic agents also known to create lung damage. However, little is known about the biological effects of low concentration oxidant gases on Type II cells. Our *in vitro* model appears as an original model to study this problem.

Isolation of Type II cells was performed using enzymatic digestion of guinea-pig lungs. However, using guinea-pigs implied modification of classical methods of selection and isolation of Type II cells because of the presence of a high percentage of inflammatory cells (namely eosinophils and neutrophils) in the lower respiratory tract of guinea-pigs when compared to rats. In addition, lung lavage from guinea-pigs contained a higher number of cells, particularly of alveolar macrophages, than lung lavage from rats, and thus permitted comparative studies of autologous macrophages and Type II cells. The selection of Type II pneumocytes was performed by a unit gravity velocity sedimentation procedure on a discontinuous gradient of Percoll. Type II cells were best identified by the presence of lamellar inclusion bodies, as seen by transmission electron microscopy. Although the modified Papanicolaou stain was the most practical method for determining Type II cells in cell separation procedures, only May-Grunwald stain could be used when cells were observed on triacetate cellulose membrane. Using these methods the purity of Type II cell preparation ranged from 80–90% in agreement with previously reported studies [4–6, 10]. However, in some experiments, we were unable to obtain a sufficient number of viable Type II cells after a 24 h culture in aerobiosis. This may be due, in part, to the effects of proteolytic enzymes like trypsin [26]. Another hypothesis involves the toxic role of polymorphonuclear cells, in particular eosinophils [27, 28]. Consistent with this hypothesis is the finding that a high percentage of polymorphonuclear cells is associated with significant cell death.

Our *in vitro* model of Type II cell culture in aerobiosis was adapted from the one previously described for alveolar macrophages [16]. This model led us to study the effects of various oxidant injuries on alveolar macrophages [29, 30]. However, there are large differences between alveolar macrophages and Type II pneumocytes. Thus, there was a need to study the effect of aerobiosis on cell behaviour, in particular on the antioxidant system. Significant activities of antioxidant enzymes were found in Type II cells. SOD activity in particular was higher than in alveolar macrophages. Type II cell cultures were studied for as long as six days which was well-suited to the study of the biological effects of toxic gases. The current study documented significant changes in antioxidant cell equipment during aerobiosis. SOD and catalase activities significantly decreased during the first three days. Glutathione cell content was strictly correlated with cell survival in aerobiosis suggesting an important role for the antioxidant system in the protection against oxidant gases and explaining why *in vitro* studies have to take into account spontaneous changes of antioxidant cell activities, especially during short-term exposure to gases.

The addition of lung extracts and/or lung explants to nutrient media did not significantly improve cell culture. Although SOD content was higher in such conditions, cell survival in aerobiosis and glutathione cell content were not modified. In contrast, the addition of GSH at the time of isolation was associated with an increase in glutathione content at a 3 day culture and a better survival in aerobiosis. There is evidence that GSH constitutes the major source of low molecular weight thiol in mammalian tissue and plays a major role in protection against oxidative injury. Thus a decreased GSH content is associated with an increased cell injury due to free radicals and peroxides. Recent studies demonstrated the presence of a Na^+ dependent uptake mechanism for GSH which supported the hypothesis that, like epithelial cells of the intestine and kidney, alveolar Type II cells can utilize plasma GSH for detoxification processes [31]. In this context, our results suggest that exogenous GSH may protect Type II cells [31] and allow a better viability in culture.

Finally, the Type II cell culture in aerobiosis was characterized by the formation of domes (fig. 4D) which are thought to result from active solute (sodium) transport from medium to substratum [32–34]. The dome formation of primary cultured monolayers of pulmonary Type II alveolar epithelial cells have been used to study the effects of nitrogen dioxide (NO_2) on the monolayer [35]. The finding that Type II cell cultures in aerobiosis formed domes suggests that an active transport by monolayer occurred after a 3 day and a 6 day culture in aerobiosis. This method might permit a quantitative evaluation of Type II cell injury by gases, as well as a morphological assessment of this injury, in a controlled local environment mimicking the alveolar epithelial environment. It is hoped that subsequent use of this model might facilitate the study of Type II injury in the pathogenesis of various chronic lung disorders.

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RÉSUMÉ: Ce travail décrit une technique d'isolement des pneumocytes II de cobaye et analyse le comportement de ces cellules en culture en aérobiose, avec une attention particulière pour l'évolution de leur équipement antioxydant. Les cellules ont été isolées par digestion enzymatique du tissu pulmonaire et sélectionnées en gradient de densité en Percoll de façon à obtenir des préparations comportant 80 à 90 % de pneumocytes granuleux, identifiés en microscopie optique (coloration de Papanicolaou) ou électronique. Les pneumocytes granuleux ont été cultivés sur membrane poreuse reposant sur un réservoir rempli de liquide nutritif, selon une méthode originale assurant un contact direct entre les cellules et l'atmosphère environnante, dans des conditions identiques à celles existant au niveau des espaces aériens alvéolaires. L'équipement antioxydant a été évalué au moment de l'isolement des cellules et après trois et six jours de culture: les activités enzymatiques, superoxyde dismutase et catalase, baissaient pendant les trois premiers jours, puis se stabilisaient entre le 3e et le 6e jour de culture; la baisse du contenu en glutathion s'accompagnait d'une mort cellulaire rapide, alors que son maintien était significativement corrélé avec la vitalité de la préparation cellulaire, évaluée par son contenu en ATP ($r=0,79$ pour $n=18$; $p<0,001$). Ce procédé de culture en aérobiose et la connaissance de l'évolution de l'équipement cellulaire antioxydant ouviant nouvelles possibilités d'étude des effets des gaz toxiques, et particulièrement des oxydants à faible concentration, sur les pneumocytes granuleux, dans des conditions expérimentalement contrôlées.