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# Human tracheal epithelial cells in culture: a suitable model for testing the cytocompatibility of materials for endotracheal use

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It is well known that cuffs of endotracheal tubes can induce ischemic injuries on tracheal epithelium, as a result of mechanical hyperpressure caused by the cuff on the airway tissue. Whether or not material components are leached out and may provoke a direct toxic effect on the respiratory epithelium is much less clear. To study the cytocompatibility of such materials, we have developed an *in vitro* cell system using human tracheal epithelial cells, arising from trachea superficial biopsies. In culture, cells have been characterized by morphological and immunocytochemical criteria. Ultrastructural observations suggest that our culture conditions are permissive for the expression of both squamous and secretory phenotypes. We have assessed the cytocompatibility of a cuff towards epithelial cells, first, by an indirect test, and second by a direct test. By the indirect test, using material extracts, we did not find any toxic effect towards human airway epithelial cells of the cuff components. By a direct test, we found a slight cell lysis after a 24 h incubation. Our study shows that this human tracheal epithelial cell system is a useful and relevant model which could be used in a quality control procedure for testing the cytocompatibility of materials for endotracheal use.

## 1. Introduction

That tracheal tube cuffs cause lesions of the trachea is a well-known phenomenon, and a wide variety of lesions of the larynx and the trachea have been reported following the prolonged use of endotracheal tubes [1–4]. The most frequently cited cause is excessive cuff-to-tracheal-wall pressure [1, 5–7]. Various modifications of the tracheal tube cuff have been made to reduce its potential for injury and it is now generally accepted that the most effective way of preventing tracheal injury is to use a tube with a smaller outer diameter along with a large residual-volume, low-pressure cuff [8]. Whether or not material components are leached out and may provoke a direct toxic effect on the respiratory epithelium in addition to the ischemic conditions, is less clear. In the biomaterial field and using assays developed in cell biology, it is possible to establish a series of cytocompatibility test methods on the basis of existing national and international testing standards, in order to study the cell response at the cell–biomaterial interface. In this respect, the use of human differentiated cell cultures provides a useful experimental model to test biomaterials in so far as the following rule is carried out: *in vitro* testing of a potential biomaterial in relation to the human cell system(s) which is (are) characteristic of the tissue(s) it will confront *in vivo* [9]. Thus, in our laboratory, primary isolated cells relevant to the function of the biomaterial are employed. Examples are human endothelial cells for seeding of vascular prostheses or human connective tissue cells such as chondrocytes or osteoblasts [10] for studying cell interaction with orthopaedic implants.

As human respiratory epithelial tissues are a prime target for airborne environmental pollutants and carcinogenesis, *in vitro* models of epithelial cells from rodent and human airways have been developed. They are of considerable interest to study the biochemical and molecular details of respiratory epithelium as well as the mucociliary transport mechanism [11–13], but also to investigate control of growth [14], squamous differentiation [15], metabolic activation [16] and effects of carcinogens [17].

However, to date, human tracheal epithelial cells in culture have never been utilized in cytocompatibility studies. Thus the aim of this work was, first, to obtain and to characterize human tracheal epithelial cells arising from adult trachea. Second, in an attempt to prove that tracheal cells can be used in an *in vitro* assay, we utilized this model for the cytocompatibility

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evaluation of a tracheal tube cuff. The possible toxicity of a material towards cells it will confront *in vivo* can be assessed by direct tests (the material to be tested comes into direct contact with cells) and by indirect tests, through the medium of material extracts [1], a method which allows study of the effect of possible toxic substances leached from the cuff. These substances were obtained under defined conditions.

## 2. Materials and methods

### 2.1. Tissue procurement and cell culture methods

Specimens of human tracheal tissue were obtained during the course of broncho-fibroscopies: superficial biopsies were removed with tweezers in a normal macroscopic area at the middle level of the trachea. The biopsies were immediately placed in F12 Coon's modification medium (Sigma Chemical Company, St. Louis, MO) and transported to the laboratory. The tissue specimens were allowed to sit overnight in the above medium at 4 °C. The explants, approximately 1 mm diameter, were placed onto the bottom of 35 mm Petri dishes (Nunc, Roskilde, Denmark) coated as follows with collagen (Sigma type VI). The collagen was diluted ($1\ mg\,ml^{-1}$) in 0.2% acetic acid, and 0.8 ml of this solution was applied in each Petri dish. The plates were gently shaken, then incubated for 3 h at room temperature in a laminar flow hood. The excess of collagen was removed and the coating was cross-linked by further incubation overnight in the presence of ammonia vapours provided by a separated sterile tank containing a 10% (w/v) ammonium hydroxide solution, at room temperature. Then collagen coated dishes were rinsed with phosphate-buffered saline (PBS), allowed to air dry in the laminar flow hood and stored at 4 °C until use. The explants (4–6 per dish) were left to attach for 15 min at room temperature before the addition of culture medium (DME/F/12, Sigma) containing $8\ g\,l^{-1}$ D-glucose, penicillin ($200\ U\,ml^{-1}$), streptomycin ($200\ \mu g\,ml^{-1}$, fungizone ($2.5\ \mu g\,ml^{-1}$) and supplemented with 2% (v/v) Ultroser G (IBF, Villeneuve-la-Garenne, France), according to [18]. Dishes were incubated at 37 °C in a humidified atmosphere (95% air) containing 5% $CO_2$. The culture medium was changed every 2 days. With this explant procedure, outgrowths consisting entirely of epithelial cells appear around the explants within 5 days. After 1–2 weeks of culture, explants were replated into new dishes to initiate additional outgrowth cultures: they can be serially replated two or three times.

Primary outgrowths were passaged when the cultures reached confluency. Cells were rinsed with $Ca^{++}$ and $Mg^{++}$-free PBS then treated with $Ca^{++}$ and $Mg^{++}$-free PBS containing 0.13% trypsin (Boehringer, Meylan, France) and 0.07% EDTA (Sigma) for 5 min at 37 °C. The dissociated cultures were suspended in culture medium containing 4% Ultroser G to inactivate the trypsin and the cell suspension was centrifuged at 800 g for 10 min. Finally, the cell pellet was resuspended in the culture medium described above and dispensed into either collagen coated culture plates or wells.

Epithelial cells can be cryopreserved, if necessary, in 90% culture medium (DME/F12, Ultroser G) and 10% dimethylsulfoxide (DMSO, Sigma).

### 2.2. Identification and characterization of epithelial cells

The epithelial nature of the cells was supported by morphological and immunocytochemical criteria.

#### *2.2.1. Electron microscopy*

At the ultrastructural level, the following techniques were used to establish the origin of the isolated cells.

*2.2.1.1. Scanning electron microscopy (SEM).* Samples were fixed for 15 min with 2% (v/v) glutaraldehyde in 0.15 M cacodylate, pH 7.3. Surfaces were then washed by 0.15 M cacodylate for 10 min. Samples were then dehydrated through a graded series of ethanol from 25 to 100%. The dehydrated samples were critical-point dried with liquid $CO_2$ and finally coated with metal using a gold target (20 nm thick), before being observed in a Hitachi S 2500 microscope.

*2.2.1.2. Transmission electron microscopy (TEM).* Samples were fixed with the glutaraldehyde-cacodylate buffer for 1 h at 4 °C. Cells were then washed in 0.15 M cacodylate. Post-fixation with 2% (v/v) $OsO_4$–0.3 M cacodylate was carried out for 60 min. The samples were dehydrated through a graded series of ethanol from 25 to 100%. The dehydration was carried out with propylene-oxide Epon (1 : 1). Finally, the samples were placed in 100% fresh Epon and polymerized in a 60 °C oven for 48 h. Sections (about 60 nm) were cut with a diamond knife and observed in a Hitachi HU 11 E transmission electron microscope.

#### *2.2.2. Keratin analysis*

Since keratins are a good marker for epithelial cells, a keratin analysis was performed.

*2.2.2.1. Indirect immunofluorescence.* Cells grown on collagen coated plates were fixed in ice-cold absolute methanol, then incubated with a first rabbit polyclonal antibody (Sanbio bv, Uden, Holland) diluted 1 : 40 in 0.1 M PBS.

After 2 h at 37 °C, cells were washed with PBS and finally incubated with the second antibody: a fluorescein isothiocyanate-conjugated (FITC) goat anti-rabbit immunoglobulin antibody (Nordic Immunological Laboratories, Holland) diluted 1 : 30 in 0.1 M PBS for 30 min at 37 °C. The cultures were washed three times with PBS, mounted and observed in an Olympus fluorescence microscope.

*2.2.2.2. Extraction of keratins.* Keratins were extracted from cells according to a procedure described by Franke *et al.* [19] and analysed by electrophoresis on a 10% (w/v) polyacrylamide gel under denaturing and reducing conditions [20].

*2.2.2.3. Immunoblot analysis.* Keratins separated by electrophoresis were transferred by passive diffusion from the gel slab to nitrocellulose. Thereafter blots were cut in strips, saturated using 3% (w/v) bovine serum albumin (BSA) in 0.1 M PBS, pH 7.4, for 3 h at 37 °C. After washing using a 0.1 M PBS, pH 7.4, containing 0.5% (w/v) BSA and 0.2% (w/v) Tween 20, blots were incubated with a monoclonal anti-epithelial $AE_1/AE_3$ mix (ICN Biomedicals, St Laurent, Quebec, Canada) diluted 1:500 in PBS-Tween-BSA overnight at 37 °C. Strips were washed with PBS-Tween-BSA and immunoglobulins fixed were revealed by an incubation of a peroxidase-labeled goat anti-mouse antibody (Institut Pasteur, Paris, France) diluted 1:1000 in 0.1 M PBS, pH 7.4 for 1 h at 37 °C.

Immunoglobulins were developed using a peroxidase solution: chloronaphtol (3 mg ml$^{-1}$ of methanol) in 0.1 M PBS, pH 7.4, containing 0.02% (v/v) $H_2O_2$. The reaction was stopped with 0.1% (w/v) sodium dodecyl sulfate.

## 2.3. Material under test

The material used was polyvinyl chloride (PVC) constituting the low-pressure cuff of a currently used endotracheal tube (Portex Limited, Hythe Kent, England), the physical characteristics of which have already been studied [8]. The material had been sterilized using ethylene oxide.

## 2.4. Indirect tests

### 2.4.1. Obtention of material extracts

The indirect cytocompatibility study is subordinate to the obtention of possible released products from the material under defined conditions [standard AFNOR, 21]. Fragments of sterile material were immersed in different extraction vehicles, the ratio of the sample surface to the volume of the vehicle being 5 cm$^2$ ml$^{-1}$. Four extraction vehicles were used:

(a) culture medium alone (DME);
(b) culture medium containing 50% artificial saliva (AS), the composition of which is 12 mM NaCl, 17.8 mM $NaHCO_3$, 16 mM KCl, 2 mM $NaH_2PO_4H_2O$, 1.5 mM $KH_2PO_4$, 3.4 mM KSCN [22];
(c) culture medium containing 10% bronchial mucus (BM): mucus samples were collected from seven randomized patients during routine bronchial toilet by assisted cough or aspiration through their tracheostomies, then pooled and dispersed in culture medium before being sterilized through 0.22 μm filtration units (Millipore, Bedford, MA);
(d) culture medium containing 50% gastric liquid (GL): samples were collected from five randomized patients during the course of endoscopy for ulcers before therapy then pooled and added to the culture medium before being sterilized through 0.22 μm filtration units.

Extractions were performed in borosilicated glass tubes at 37 °C for 120 h without agitation, according to the standard. At the end of the incubation period, fragments of materials were removed and the so-called extracts were used. Borosilicated glass tubes containing identical extraction vehicles without any material were processed under the same conditions to provide controls.

### 2.4.2. Material examination

Fragments of material were observed by SEM analysis, before the extraction procedure and also after their removal from the extract containing 10% BM.

### 2.4.3. Contact between cells and materials extracts

Epithelial cells were seeded at a density of $10^5$ cells cm$^{-2}$ in 96 well assay collagen coated plates (Nunc) and the cultures were maintained at 37 °C for 48 h after cell plating. The medium was removed and replaced by material or control extracts at various concentrations (100%, 50%, 10%, 1%) in the culture medium for 3 days at 37 °C. In order to include a positive control of cytotoxicity, other epithelial cells were submitted to 0.2 mM sodium arsenite.

### 2.4.4. Cell metabolic activity assay: the MTT assay

The assay is based on the observation that a mitochondrial enzyme of viable cells have the ability to metabolize a water-soluble tetrazolium dye 3–(4, 5–dimethylthiazol–2–yl)–2, 5–diphenyl tetrazolium bromide (MTT: MethylThiazol Tetrazolium) into an insoluble formazan salt [23]. At the end of the extract incubation period, material or control extracts were discarded, cell layers rinsed twice in PBS, and MTT in PBS (125 μl per well of a 5 mg ml$^{-1}$ MTT solution) was added for 3 hours at 37 °C. At the end of this time, MTT solution was removed and the insoluble formazan crystals formed were dissolved in 100 μl DMSO.

Finally, the absorbance was measured at 540 nm, using an enzyme-linked immunosorbent assay microtitre plate spectrophotometer. The intensity of the blue colour obtained is directly proportional to the metabolic activity of the cell and inversely proportional to the toxicity of the extract.

## 2.5. Direct test

A variety of test methods have been recorded for the assessment of altered cellular functions and characteristics caused by biomaterials. One characteristic, the integrity of the cell membrane, can be evaluated by the

determination of cell membrane permeability to intracellular enzymes. Lactate dehydrogenase enzyme (LDH) is found in the soluble cell fraction so that any agent which damages the cell surface will cause a release of this enzyme into the supernatant. This technique has been developed to screen biomaterials using macrophages and synovial fibroblasts [24].

In the present study, we quantified the release of LDH from epithelial cells in direct contact with PVC for 4 h and 24 h. Samples of PVC were discs of 15.5 mm diameter spread over the bottom of the wells of four-tissue culture plates (Nunc). The test was carried out with human tracheal epithelial cells. Cells ($4 \times 10^4$ cells $cm^{-2}$) were plated into empty wells (negative control, NC: they will contain the spontaneous release), as well as in wells containing discs of material (PVC), and plates were incubated for 4 h and 24 h under 5% $CO_2$ at 37 °C.

The LDH maximal release and the control of non-specific LDH binding, after release, to the wells and to the materials was assessed by using the cell suspensions after five repeated freeze/thaw cycles: lyzed cells were then plated in wells (called positive control (PC) wells) and incubated for the same times. At the end of the incubation periods, the supernatant from each well was withdrawn, centrifuged (800 g, 10 min) and assayed for LDH activity (kinetic determination by a spectrophotometric assay using a commercial kit: Enzyline, bioMérieux, Marcy l'Etoile, France) at 37 °C as recommended by the German Society for Clinical Chemistry. The LDH activity of fresh medium was subtracted from all samples. The recorded values were expressed as units per litre ($U\,l^{-1}$). 5 parallels were run in each test series.

### 2.6. Expression of data and statistical analysis

Concerning indirect tests, results following incubation of cells with extracts were available as a mean value of absorbances obtained from cells incubated in the presence of the extracts whether they were control or material extracts. The percentage of viable cells was calculated as follows

$$\frac{\bar{E}}{\bar{C}} \times 100$$

for each extract at a given concentration in which $\bar{E}$ is the mean absorbance obtained from material extracts ($n = 8$), $\bar{C}$ is the mean absorbance obtained from control extracts ($n = 8$).

The standard error of the percentage was calculated using a formula [24] derived from [25]

$$S \approx \left[ S_E^2 \frac{1}{\bar{E}^2} + S_C^2 \frac{\bar{E}^2}{\bar{C}^4} \right]^{1/2} \times 100$$

where $S$ is the standard error of the ratio $\bar{E}/\bar{C}$, $S_E$ is the standard deviation in the calculation of $\bar{E}$ and $S_C$ is the standard deviation in the calculation of $\bar{C}$.

Concerning direct tests, cell damage can be quantified in terms of a percentage of LDH release

$$\Delta = \frac{(\text{U/l})\ \text{PVC} - (\text{U/l})\ \text{NC}}{(\text{U/l})\ \text{PC} - (\text{U/l})\ \text{NC}} \times 100$$

where (U/l) PVC corresponds to the values recorded from the supernatants of wells containing cells plus material, (U/l) NC corresponds to the values recorded from the supernatants that contain the LDH spontaneous release and (U/l) PC corresponds to the values recorded from supernatants that contain the LDH maximal release.

The student's $t$-test was used for statistical analysis.

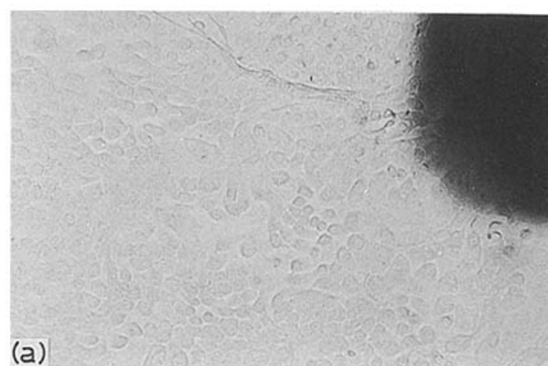
## 3. Results

### 3.1. Epithelial cell culture

Tissue specimens obtained were suitable for initiating outgrowth of cultures. Almost 70% of the explants produced pure epithelial outgrowths.

As early as 4–5 days after plating, a rapidly enlarging ring of epithelial cells appeared around each explant (Fig. 1a). Some ciliated cells were present in the outgrowth during the first week after initiation of the culture. The outgrowth continued to extend and cells grew as a coherent sheet until confluency was reached 2–3 weeks later (Fig. 1b). Fibroblasts were absent from cultures and the monolayer obtained appeared to be entirely constituted of epithelial cells without any overlapping between cells. The epithelial cells can be successfully passaged in collagen-coated culture wells. The passaged cells attached readily to the coated plastic surface.

If necessary, human tracheal epithelial cells could be successfully cryopreserved. After thawing, cell viability determined by exclusion of Trypan blue was over 90%, and cells could be seeded and used.



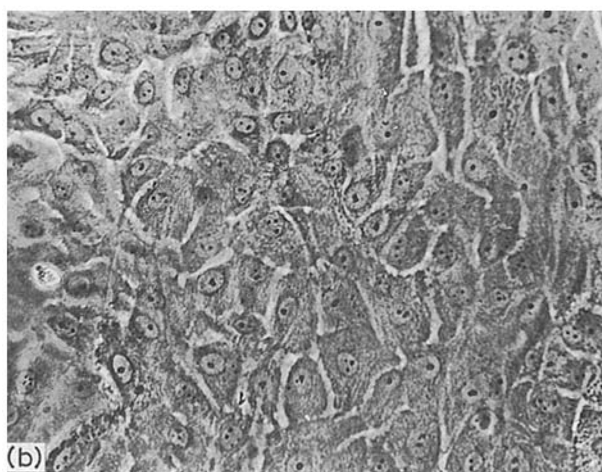




*Figure 1* (a) Primary outgrowth culture near the edge of the explant, × 100; (b) monolayer of human tracheal epithelial cells, × 200.

### 3.2. Characterization of epithelial cells

Under the inverted light microscope, when the monolayer was obtained, uniform fields of cells with closed cell-to-cell contacts were observed (Fig. 1b).

#### 3.2.1. *Electron microscopy*

At the ultrastructural level, the isolated cells showed several features typical of epithelial cells. Scanning electron micrographs of the outgrowing culture showed surface specializations such as numerous microvilli (Fig. 2a, b). In Fig. 2c, two classes of cells were discernible: small cells merged in a focus in the upper right quadrant of the micrograph surrounded by large, probably squamous, cells. Fig. 2d shows a scanning electron micrograph of ciliated cells in the outgrowth.

The TEM studies showed that the cells were interconnected by typical desmosomes (Fig. 3a, b).

Many tonofilament bundles were found in the cytoplasm in close relation to desmosomes and protruding from them (Fig. 3c) as well as around nuclei (Fig. 3d). The cytoplasm contained secretory granules and mitochondria (Fig. 3e). The cornification process, as described by Jetten *et al.* [26] probably occurred in these cultures, as shown by the cross-linked envelope (Fig. 3f, g): the electron dense cell membrane also called electronlucent cornified cells by these authors.

#### 3.2.2. *Keratin analysis*

All cells in the culture reacted positively with the polyclonal keratin antibody, as visualized by immuno-

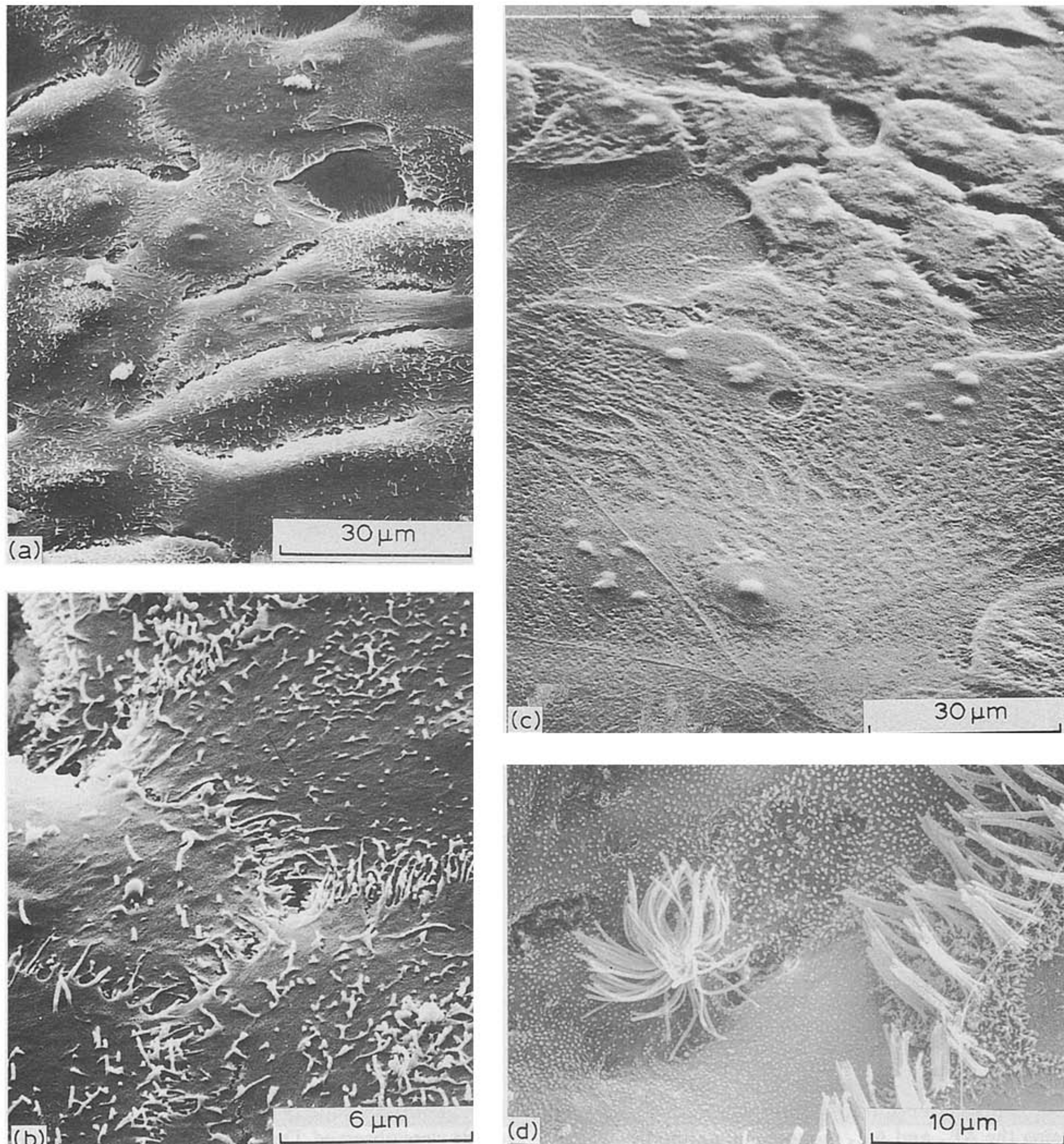



*Figure 2* Epithelial cell characterization. (a, b) SEM of cell surfaces showing abundant microvilli; × 1000 and × 5000, respectively. (c) SEM showing that some fields are comprised of a mixture of small cells grouped in foci (upper right quadrant) and surrounding larger cells; × 1000. (d) SEM showing ciliated cells in the outgrowth; × 3000.

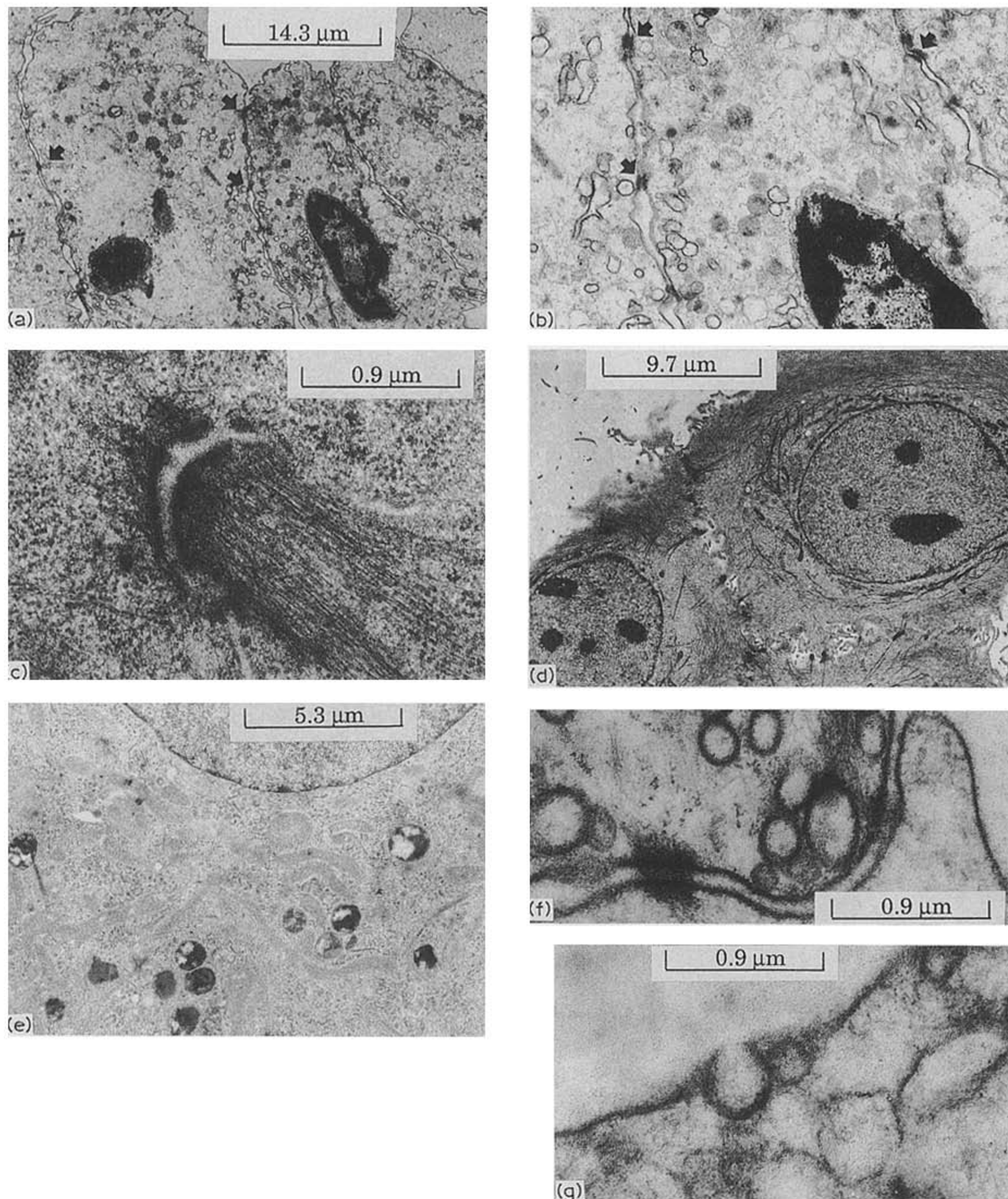



*Figure 3* Transmission electron micrographs. (a, b) Cells are interconnected by typical desmosomes; × 2100 and higher magnification, respectively: arrows point to the desmosomes. (c) Desmosome associated with tonofilaments; × 33600. (d) Tonofilament bundles around nuclei; × 3100. (e) Some cells are replete with secretory granules and mitochondria; × 5600. (f, g) Cross-linked envelope of typical cornified cells; × 33600.

fluorescence staining (Fig. 4). To examine the expression of keratins, keratin-enriched fractions were prepared: identical protein amounts (15 μg/$10^5$ cells) were extracted from cells cultured in the presence of 50% (v/v) DME control or material extract and separated by gel electrophoresis (Fig. 5a). The bands were then analysed by immunoblot procedure. Immunoblot analysis of cell fractions (Fig. 5b) was performed using the $AE_1$ and $AE_3$ monoclonal antibodies which are known to react with the acidic type **I** keratins and the basic type **II** keratins, respectively. The results of these studies show that epithelial cells expressed 40–43–46–48–50–54–56 kilodalton proteins and the $AE_1/AE_3$ monoclonal antibody mix recognized all the bands transferred except the 43 kD one, thus confirming the other bands as keratins. The 43-kD protein did not react with the $AE_1/AE_3$ antibodies and was therefore not positively identified as a keratin. As shown in Fig. 5, no changes in keratin expression related to the presence of the material in the extraction vehicle were observed. Denser bands observed for cells in the presence of control extracts (C), compared with those of material extract (M) are the results of loading different amounts of antigens on the gel.

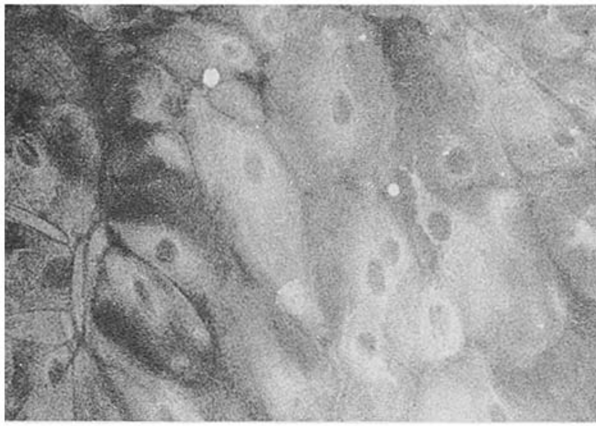


*Figure 4* Indirect immunofluorescence staining of keratins; × 200.

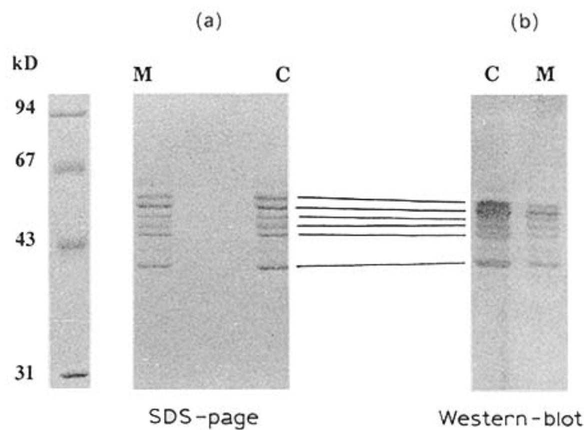



*Figure 5* Keratin expression in human tracheal epithelial cells. (a) Keratin profile of epithelial cells cultured in the presence of 50% (v/v) DME control (C) or material (M) extract. (b) Immunoblot analysis of keratins expressed by the same cultures using the $AE_1/AE_3$ monoclonal antibody mix to identify acidic and basic kerains, respectively. Numbers refer to the molecular weight markers: phosphorylase b (94 kD), BSA (67 kD), ovalbumin (43 kD), carbonic anhydrase (31 kD).

### 3.3. Cytocompatiblity study

The surface state of the PVC cuff was studied by SEM. Before any manipulation (Fig. 6a, b), SEM showed surface irregularities the size of which range from 0.2–1 μm. The PVC was also examined after it had been immersed for the 5-day extraction period in the 10% (v/v) BM extract. The surface state is completely different (Fig. 6c, d), and appears smoother than the previous state. The presence of adsorbed protein layers from the extraction vehicle to the PVC surface could hide the irregularities and explain the polished appearance depicted in Fig. 6c, d.

Concerning indirect tests, two sets (material or control) of four different extracts were tested on confluent epithelial cells. The percentage of viable cells was calculated for each extract at a given concentration and results are presented in Fig. 7. No significant differences in cell metabolic activities were observed, since the MTT assay allows quantification of not only cell viability but also cell metabolic activity in relation to the activity of a mitochondrial enzyme: the succinate dehydrogenase. The mean absorbance obtained for cells submitted to 0.2 mM sodium arsenite was 0.030, which corresponds to less than 10% cell viability. A cell viable percentage over 75% is required in the standard [27] to rule out any cytotoxic effect of the extracts on the cultured cells, so that none of the extracts tested were cytotoxic. Moreover, as already mentioned, the molecular weights of keratins obtained from cells cultured in the presence of DME cuff extract were identical to those obtained from cells cultured in the presence of DME extract without material (Fig. 5).

Concerning direct tests, LDH release from cells is shown in Fig. 8 and Table I. The maximal release was obtained after repeated freeze/thaw cycles and there was no non-specific LDH binding. Whatever the incubation time considered, LDH release from cells in contact with material (PVC) was significantly higher than LDH release obtained from control cells (NC): $p < 0.01$ at 4 h and $p < 0.001$ at 24 h. Cell membrane injury provoked by the biomaterial can be expressed as a percentage of LDH release (Table I). The percentages of LDH release obtained at 4 h and 24 h were significantly different ($p < 0.05$). The percentage of LDH release obtained at 24 h seems to indicate a slight cell lysis.

## 4. Discussion

*In vitro* testing procedures are of considerable importance in the biomaterial field because they constitute a first necessary step before the *in vivo* evaluation. Cell culture is a very sensitive technique which is highly reproducible, allowing a qualitative, quantitative and statistical approach. The aim of the present work was to assess the cytocompatibility of the components of a cuff of an endotracheal tube currently used in intensive care units for respiratory assisted patients.

For several years, studies performed to estimate cyto or biocompatibility have been generally limited to semi-quantitative cytotoxicity tests using poorly differentiated cell lines, sometimes transformed, mainly of neoplastic origin. Taking into consideration the current application of low-pressure cuffs, we have chosen to carry out the experiment on human differentiated cells (tracheal epithelial cells) which are involved in the synthesis and maintenance of a highly specialized function. Differentiated human models have a phenotype expression which must be constantly controlled *in vitro*. Thus, we established and characterized tracheal epithelial cell cultures arising from samples taken from normal macroscopic areas.

In our culture conditions, ultrastructural observations of human tracheal cells supported the functional epithelial nature of the cultured cells. The expressed characteristics were in accordance with several aspects previously described in human [28] or animal [26, 29] models, including microvilli on cell surfaces, desmosomes between cells, tonofilaments in the cytoplasm, and synthesis of keratins. Ciliated cells have always been observed during the first week of the cultures. Epithelial cells for cytocompatibility studies were used in the first or second passage and we did not observe any more ciliated cell differentiation in the passaged cultures. The ciliated cell is one of the main cell types

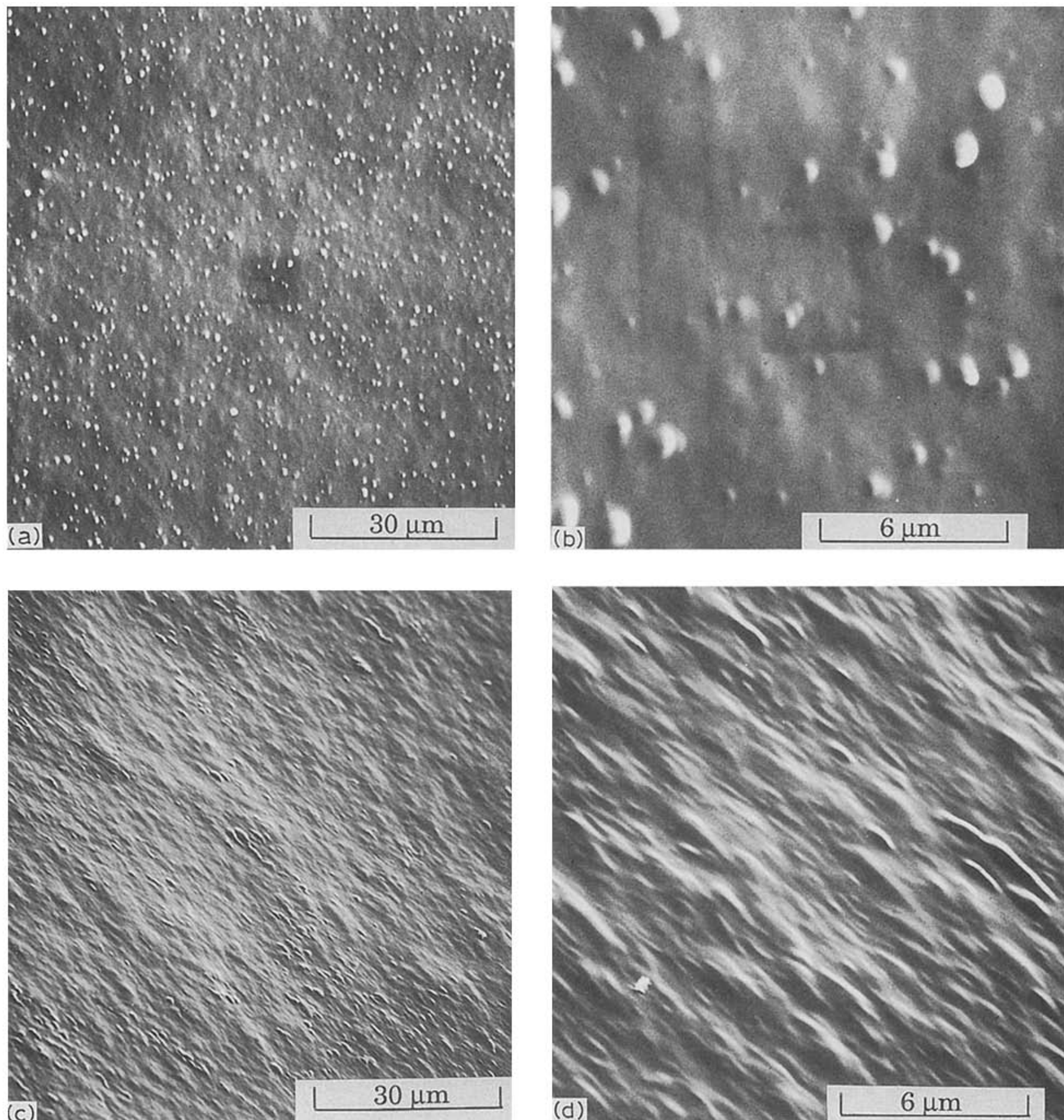



*Figure 6* Scanning electron micrographs of PVC: (a, b) before any manipulation; × 1000 and × 5000, respectively and (c, d) after removal from the extraction vehicle containing 10% (v/v) BM, in which PVC has been immersed for 5 days at 37 °C; × 1000 and × 5000, respectively.

of the epithelium of the trachea *in vivo* and is probably a non-proliferative, terminally differentiated cell [26]. The non-ciliated population consists of a variety of secretory cells and a non–secretory cell, the basal cell [26]. It is known that normal epithelial cells cultured in the absence of retinoids no longer proliferate and undergo terminal differentiation into populations of non-proliferating squamous cells. The results of TEM studies seem to indicate that the culture conditions we have used are permissive for the expression of both squamous and secretory phenotypes. Morphological signs of the squamous differentiated phenotype are exhibited by the great number of keratin filament bundles, many desmosomes associated with keratin filaments and the presence of a cross-linked envelope, an electron dense cell membrane as depicted by Jetten *et al.* [26], indicating a typical cornification process. The expression of the secretory phenotype is corroborated by the presence of numerous secretory granules in the cytoplasm.

The epithelial cultures we have propagated on a collagen-coated plastic surface exhibit in some portions at least two cell subpopulations, and could be, according to Ke *et al.* [30], a mixture of small dividing cells grouped in foci and surrounded by large squamous cells.

The present work is the first to our knowledge which reports the use of such a model for the purpose of biocompatibility studies. Generally, tracheal epithelial cell and organ culture, arising either from humans or animals, has been and is effectively utilized to study the biochemical and molecular details of

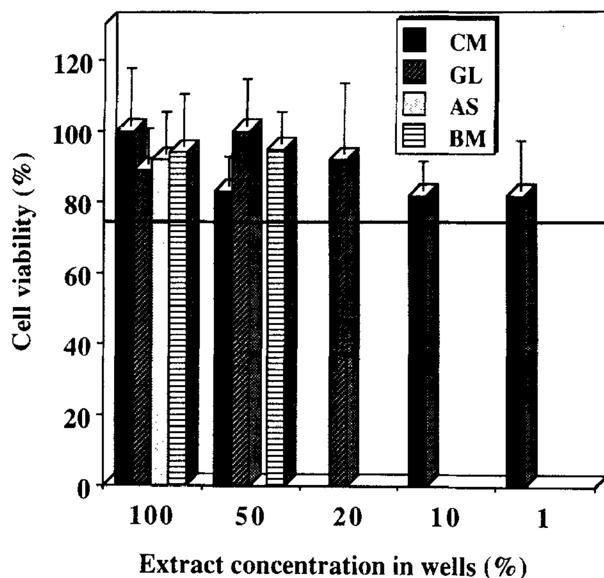



*Figure 7* Percentage of cell viability after a 3-day incubation period between human confluent epithelial cells and material or control extracts. The following extracts were tested: culture medium alone (CM), culture medium containing artificial saliva (AS), culture medium containing bronchial mucus (BM), and culture medium containing gastric liquid (GL). The thick line represents the cell viable percentage above which non cytotoxic effect is observed.

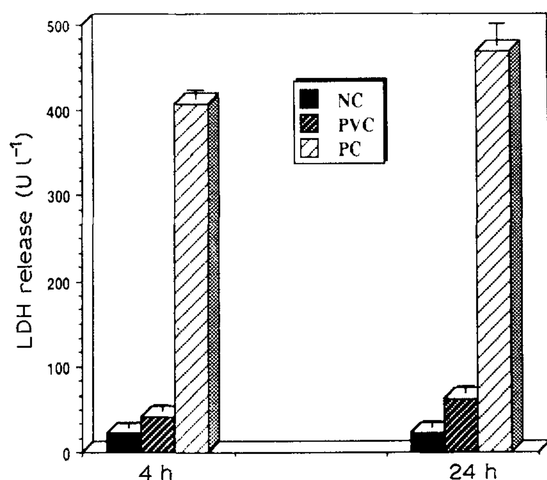



*Figure 8* LDH release from human tracheal epithelial cells in direct contact with PVC for 4 and 24 h: NC represents the LDH mean value recorded from the supernatants in negative control wells (it represents the spontaneous release); PVC represents the LDH mean value recorded from the supernatants in wells containing the material; PC represents the LDH mean value recorded from the supernatants in positive control wells (it represents the LDH maximal release obtained from lyzed cells).

respiratory tract epithelium as well as the effect of environmental toxicants. On the basis of existing national testing standards, we have assessed the cytocompatibility of a cuff towards epithelial cells, first, by an indirect test, second, by a direct test. By the indirect test we did not find any toxic effect of the possible substances leached from the cuff in the different extraction vehicles. By the direct test and the measurement of LDH release from epithelial cells in direct contact with the material, we found a slight cell lysis after a 24 h incubation. Further investigations in order to characterize the squamous differentiation (increase in transglutaminase activity and cholesterol sulfate levels, induction of cross-linked envelopes) as well as the synthesis and secretion of glycoproteins, are in progress at the interface epithelial-cell–biomaterial in our laboratory.

TABLE I LDH activity, expressed as Units/litre, recorded from centrifuged supernatants, that have been in contact for either 4 h or 24 h with human tracheal epithelial plated into negative control (NC) wells (the value represents the spontaneous release) and into wells containing material (PVC). Moreover, the LDH maximal release (positive control: PC) was assessed from cells that have been damaged by freeze/thaw cycles before being plated in wells for the same incubation times

| Series | Incubation time (h) 4 | 24 |
|---|---|---|
| Negative control (NC) $n = 5$ | $22 \pm 4.9$ | $23 \pm 8$ |
| Positive control (PC) $n = 3$ | $408 \pm 22$ | $470 \pm 38$ |
| Material (PVC) $n = 5$ | $41 \pm 9$ $p < 0.01$ | $62 \pm 8.6$ $p < 0.001$ |
| $\Delta \frac{PVC - NC}{PC - NC} \times 100$ | $4.9 \pm 2.4$ | $8.7 \pm 1.6$ |

Our study shows that this human tracheal epithelial cell system, which retains many morphological and functional characteristics of the cells *in vivo*, could be a suitable model for a quality control and a regulation pre market approval of materials for endotracheal use.

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