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Evaluation of the human cell line RPMI 2650 as an *in vitro* nasal model*†

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SUMMARY

We have investigated a human nasal cell line, RPMI 2650 (ATCC), as an in vitro model to assess the absorption and tolerability of nasally administrated peptides. This cell line was cultured on different filters (i.e. Nunc, Costar, Falcon, Millipore, and Becton) which were uncoated or coated with collagen types I or IV, laminin, fibronectin and extracellular matrix. Cell line morphology, capability of forming a cell layer and phenotype were analyzed by light-, fluorescence- and electron microscopy. The morphological analysis showed that RPMI 2650 cells were forming cell clusters on one group of filters (i.e., Nunc, Costar, Falcon and Becton filters coated with collagen types I or IV, laminin and extracellular matrix). On the second group of filters (i.e., Becton and Millipore filters, coated with fibronectin) the cells had the tendency to spread in a cell layer. In both groups of filters, the cells never showed cell polarization, nor microvilli and tight junctions. Phenotyping of this cell line was performed by indirect immunofluorescence using monoclonal antibodies against human cytokeratins 10, 17 and 18 (markers of epithelial cells), desmin (marker of mesothelial cells) and vimentin (marker of mesothelial cells). Vimentin was strongly expressed, cytokeratins 10, 17 and 18 were weakly expressed, and desmin was not expressed. The human nasal cell line RPMI 2650 was not able to form a tight cell layer under these cell culture conditions. The limits of this cell line as an in vitro nasal model for drug absorption is discussed.

Key words: nasal absorption, cell culture, nasal drug delivery

INTRODUCTION

The interest in nasal administration as a systemic route has increased significantly during the last decade. This is in particular the case for peptides which can otherwise only be administrated parenterally due to their extremely low oral bioavailability. The nasal route offers the advantages of a reasonable bioavailability and a fast absorption route. Up to now, the feasibility of the nasal absorption route has been assessed with nasal mucosa, mostly *in vivo* or *ex vivo*.

The rat model allows drug to be administrated quantitatively to the nasal cavity of anaesthetized rats. The areas under the blood concentration time curves after intranasal application can be compared to the intravenous administration taken as a reference route (Hirai et al., 1981). A variety of peptides with or without enhancers have been shown to be well-absorbed from the nasal mucosa with bioavailabilities (compared to intravenous or subcutaneous administration) ranging from 70-100% (Su et al., 1985; Kissel et al., 1992; Ikeda et al., 1992; Shyu et al., 1993).

In order to avoid time- and animal-consuming models, all attempts have to be undertaken to find suitable and simple alternatives in the development of *in vitro* models. *In vitro* cell culture presents many advantages including a rapid assessment of the potential permeability and metabolism of a drug, the opportunity to elucidate the molecular mechanisms of drug transport and pathways of drug degradation (or activation), a rapid screening of new drugs and the possibility to use human tissues (Audus et al., 1990). Three different types of nasal tissue culture systems are available: primary explants, organ cultures, and cell lines. We have decided to evaluate the cell line system in order to have a genetically homogenous and therefore more reproducible nasal model. Attention should be taken concerning the selection of the cell line, the microporous membrane, the supporting matrix and the culture conditions. The extracellular matrix is the natural microenvironment for the epithelial cell migration, proliferation and differentiation. The extracellular matrix is composed of mostly collagen of various

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types, glycosaminoglycans, and glycoproteins. We have investigated cell attachment, migration, proliferation and differentiation of the human nasal cell line RPMI 2650 on a whole extracellular matrix extract, two types of collagen (I and IV), and two glycoproteins (fibronectin and laminin). These cell substrates were coated on different microsporous membranes. A morphological study was performed by light-, transmission- and scanning electron microscopy, and a phenotypic characterization was carried out in parallel using fluorescence microscopy.

MATERIAL AND METHODS

Cell line

The human nasal cell line RPMI 2650 (ATCC, CCL-30) was purchased from the American Type Cell Culture Condition (ATCC), 7th Edition 1992. This cell line was derived from a pleural effusion of a patient with an anaplastic squamous cell carcinoma of the nasal septum (Moore et al., 1964; Moorhead, 1965).

Culture media

We have seeded and cultured the cells in Dulbecco's modified Eagle medium (DMEM)/Ham's F-12 (1:3; DMEM-Ham F12; Gibco BRL, Europe). This medium was supplemented with 10% Fetal Calf Serum (FCS; Gibco BRL, Europe).

Filters or tissue culture inserts

We have investigated five different filters: Nunc (Denmark), Costar (USA), Falcon (USA), Millipore (USA) and Becton (USA) coated either with collagen type I or IV, laminin, extracellular matrix or fibronectin (Table 1).

Cell culture

The cells were seeded on the 42th passage and cultured according to the ATCC guidelines for one week at 37°C, under 5% CO_2. Then, they were trypsinized and seeded in a range of 5×10^4 to 6×10^6 cells/ml on the uncoated or coated filters in DMEM-Ham-F12 containing 10% FCS until reaching confluence.

Morphology

The morphology of the cell line was assessed at first under light microscopy with a stereomicroscope. The membranes were fixed in 3% glutaraldehyde in phosphate buffer for 6-12 h at 4°C. Afterwards the membranes were removed from the filters, washed in phosphate buffer and post-fixed for 1 h with 1% phosphate-buffered osmium tetroxide. The specimens were dehydrated in a graded series of ethanol and processed for transmission (TEM) and scanning electron microscopy (SEM).

Phenotyping

The cells were grown either on multiwell slides (Labteck, Nunc, USA) or on the tested filters (Table 1) and immunolabeled when they reached confluence. The cells were washed, fixed in methanol, and incubated with the following mouse anti-human monoclonal antibodies: anti-cytokeratins 10, 17 and 18 (Dako, Denmark), anti-desmin (Immunotech, France), and anti-vimentin (Immunotech, France), used at a working dilution of 1:50. Chromatographically-purified mouse immunoglobulins G (IgG; Zymed, USA), diluted at 1:2,500, were used as a negative isotypic control. The immunolabeling was amplified with a biotinyl IgG F(ab)'2 (Zymed, USA) at a working dilution of 1:100 and visualized by a goat anti-mouse streptavidin conjugated to fluorescein (Zymed, USA), at a working dilution of 1:100. The cells were then mounted and observed under a fluorescence microscope (Leica, Switzerland).

RESULTS

Light microscopy

The attachment of the RPMI 2650 cell line was coating-dependent. The filters were divided into two groups according to the degree of adhesion and spreading on the membranes. On the membranes belonging to group 1 (Nunc, Costar, Falcon and Becton, uncoated or coated with collagen type I or IV, laminin and extracelluar matrix) the majority of cells formed smaller or larger clusters (Figure 1a). Group 2 included membranes coated with fibronectin (Becton: 13 µg/cm^2; and Millipore: 10, 20, or 40 µg/ml) on which the cells showed a tendency to spread over the whole area of the membranes (Figure 2a).

Table 1. Tested filters or tissue culture inserts.

filter	membrane	pore size (µm)	surface (mm^2)	type of coating	source of coating
Nunc	anopore inorganic	0.2	314	collagen type I collagen type IV Fetal Calf Serum	rat tail human placenta
Costar	polycarbonate	0.4	133	collagen type IV	human placenta
Falcon	cyclopore	0.45	114	collagen type IV	human placenta
Millipore	biopore	0.4	2830	fibronectin	cellular
Becton	cyclopore	0.45	1660 123 123	fibronectin laminin matrigel (extracellular matrix)	human plasma EHS mouse tumour extracted from EHS mouse tumour

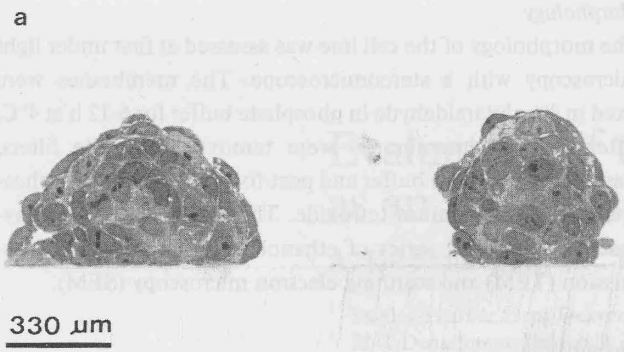


Figure 1. Morphology of the human nasal cell line RPMI 2650 cultured on Nunc filter coated with collagen type I at 1×10^6 cells/ml: a) light micrograph of cell clusters (toluidine blue staining); b) scanning electron micrograph of a cell cluster.

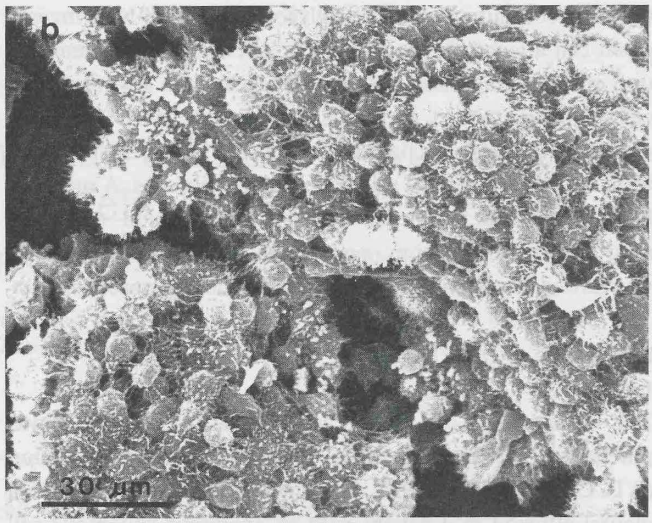
Figure 2. Morphology of the human nasal cell line RPMI 2650 cultured on Millicell filter coated with fibronectin (40 µg/ml) at 6×10^6 cells/ml: a) light micrograph of flat cells (toluidine blue staining); b) scanning electron micrograph of flat cells.

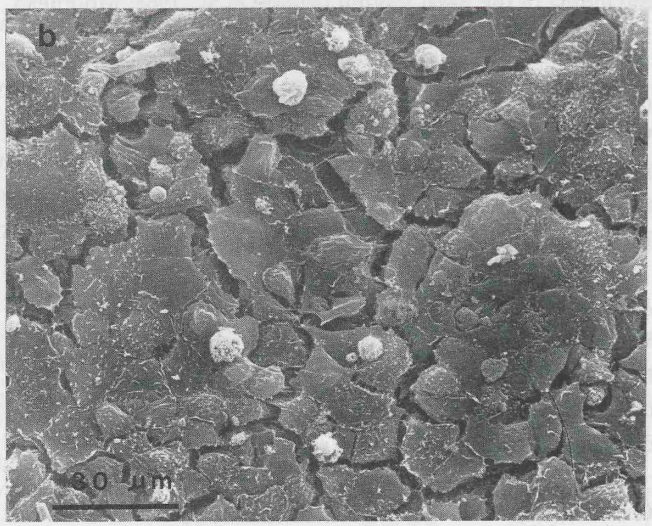
Transmission electron microscopy

In both groups of membranes the cells displayed very similar morphological characteristics. They presented a great number of mitotic figures, indicative for a high proliferation rate. The nuclei were round or slightly lobulated with prominent nucleoli, and the cytoplasm contained a great number of organelles such as mitochondria, endoplasmic reticulum and Golgi apparatus (Figure 3). The cells were not polarized (differentiation in apical and basal part) and had no cilia. The cells forming cell layers or clusters were not connected by tight junctions and showed only immature desmosomes.

Scanning electron microscopy

Scanning electron microscopical evaluation of these cells confirmed the above-described morphological features. This cell line was not growing as a monolayer. On the membranes from group 1 the cells formed mainly clusters composed of round and slightly flattened cells which were loosely arranged. The single clusters were connected by smaller or larger cell bridges. The surface of the round cells was covered with microvilli and small folds whereas the surface of the more flat cells was smooth with only a few microvilli. Cilia were missing (Figure 1b). In group 2 the cells were arranged in cell formations of different sizes. The single cells in the formation were reasonable flat and formed several layers by overlapping each other. In





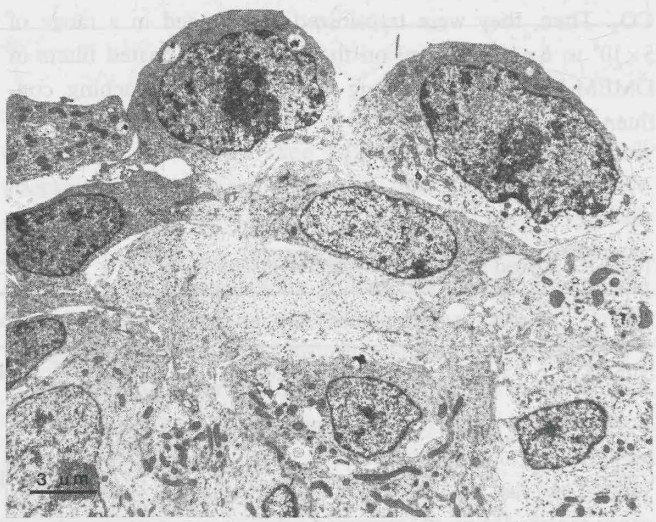


Figure 3. Ultrastructural features of the nasal cell line RPMI 2650 cultured on Nunc filter coated with collagen type I at 1×10^5 cells/ml.

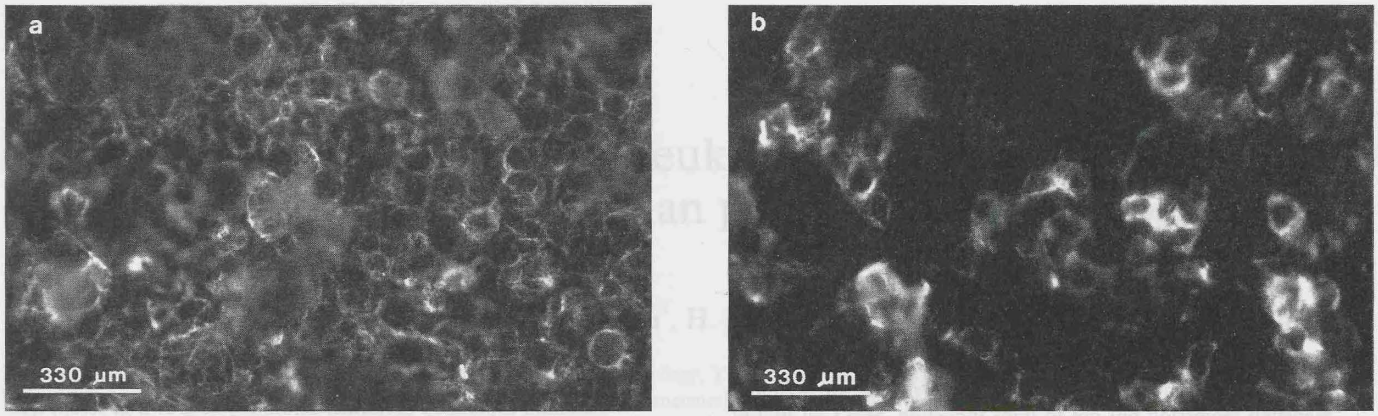


Figure 4. Immunophenotype of the human nasal cell line RPMI 2650 cultured on Millipore filter coated with fibronectin (40 µg/ml) analyzed by fluorescence microscopy: a) expression of cytokeratins 10, 17 and 18; b) expression of vimentin.

Table 2. Cytokeratins 10, 17 and 18, vimentin and desmin expression on the human nasal cell line RPMI 2650 (n=5). Indirect immunofluorescence revealed by a biotin-streptavidin-fluorescein amplification.

intracytoplasmic cell marker	monoclonal antibody	immunolabeling
cytokeratins 10, 17, 18	anti-human cytokeratins 10, 17, 18	very weak
vimentin	anti-human vimentin	strong
desmin	anti-human desmin	negative

between the formations smaller or larger gaps could be observed. The great majority of cells revealed a smooth surface with a few microvilli, but they were some cells covered with numerous microvilli and small folds (Figure 2b).

Phenotyping

The cell line expressed the following epithelial phenotype in all tested filters and tested culture conditions (Table 2). All negative isotypic controls were negative. The cytokeratins 10, 17, 18 and vimentin were co-expressed mostly on the cells that were growing in clusters (Figures 4a and b).

DISCUSSION

In vitro nasal models are mostly developed to evaluate the permeability of nasal mucosa. So far, no nasal cell lines were used as *in vitro* models for drug transport. We have investigated the human nasal cell line RPMI 2650 described by Moore et al. (1964) and Moorhead (1965) as a quasi-diploid permanent cell line derived from a pleural effusion of a patient with an anaplastic squamous cell carcinoma of the nasal septum. The metabolism of this cell line has been previously assessed at the regulation of its chloramphenicol acetyltransferase (CAT) gene under different cell culture conditions which seemed to be arginine- and citrulline-mediated (Boyce at al., 1986, 1989; Jackson et al., 1986, 1988). The aminopeptidase activity of this cell line was recently described to be comparable with the one from human nasal biopsies and thought to be a suitable *in vitro* model to study the biotransformation of peptides (Peter et al., 1992). We have investigated this cell line in terms of developing an *in vitro* nasal model for transport and tolerability of nasally applied peptides. We cultured this cell line on different microporous membranes either uncoated or coated with a whole extract of extracellular matrix, and its various components (i.e., collagen type I or IV, laminin and fibronectin). Furthermore, plasma and cellular fibronectins were tested as it has been suggested that plasma fibronectin does not result from post-translational proteolytic modification of the cellular form. Plasma and cellular fibronectins were considered as two types of fibronectins (Hayashi et al., 1981). The presence of soluble fibronectin and ß1-integrin receptors on this nasal cell line was recently reported to be essential in the binding of bacterial pathogens (Byrd et al., 1993). We have shown that RPMI 2650 cells are forming cell clusters on one group of filters coated with collagen types I or IV, laminin and extracellular matrix. On the second group of filters coated with fibronectin, the cells had the tendency to spread. On both groups of filters the cells showed very similar ultrastructural features. They were not polarized, had no cilia, no mucus production, no tight junctions and only few microvilli and small folds. The cell line RPMI 2650 contains the cytokeratins 5, 7, 8, 17, 18 and 19 but no vimentin after cell culture on plastic flasks and according to the ATCC recommendations (Moll et al., 1983).

Culturing the cell line on plastic or microporous membranes uncoated or coated with extracellular matrix, collagen type I or IV, laminin and fibronectin we have observed that this cell line co-expressed the cytokeratins 10, 17, 18 and vimentin, as revealed by indirect immunofluorescence. These results are not in contradiction with the previous reported ones from Moll et al. (1983) as this cell line grew under different cell culture conditions. The co-expression of cytokeratin (epithelial marker) and vimentin (mesothelial marker) may be related to cell culture conditions and microenvironment as it has been reported for subcultured normal human keratinocytes (Richard et al., 1990). The cell line RPMI 2650 does not express desmin (desmosomal protein). Our results suggest that this cell line expresses the transformed or undifferentiated phenotype under the described cell culture conditions and may not be considered as a suitable *in vitro* model to study transport and tolerability of nasally applied peptides. Further investigations are necessary to elucidate the differentiation of this RPMI 2650 cell line under various cell culture conditions.

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