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Research Article

In-vitro Cell Culture Models of the Nasal Epithelium: A Comparative Histochemical Investigation of Their Suitability for Drug Transport Studies

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Purpose. To evaluate different in-vitro cell culture models for their suitability to study drug transport through cell monolayers.
Methods. Bovine turbinate cells (BT; ATCC CRL 1390), human nasal septum tumor cells (RPMI, 2650; ATCC CCL 30), and primary cell cultures of human nasal epithelium were characterized morphologically and histochemically by their lectin binding properties. The development of tight junctions in culture was monitored by actin staining and transepithelial electrical resistance measurements.
Results. The binding pattern of thin-sections of excised human nasal respiratory epithelium was characterized using a pannel of fluorescently-labelled lectins. Mucus in goblet cells was stained by PNA, WGA and SBA, demonstrating the presence of terminal N-acetylglucosamine, N-acetylgalactosamine and galactose residues respectively in the mucus of human nasal cells. Ciliated cells revealed binding sites for N-acetylglucosamine, stained by WGA, whereas Con A, characteristic for mannose moieties, labelled the apical cytoplasm of epithelial cells. Binding sites for DBA were not present in this tissue. Comparing three different cell culture models: BT, RPMI 2650, and human nasal cells in primary culture using three lectins (PNA, WGA, Con A) as well as intracellular actin staining and transepithelial electrical resistance measurements we found, that only human nasal epithelial cells in primary culture showed differentiated epithelial cells, ciliated nasal cells and mucus producing goblet cells, which developed confluent cell monolayers with tight junctions.
Conclusions. Of the in-vitro cell culture models studied, only human nasal cells in primary culture appears to be suitable for drug transport studies.

KEY WORDS: nasal cell culture models; differentiation; histochemical characterization; lectin binding.

INTRODUCTION

The nasal route has attracted considerable interest for the delivery of proteins and peptides recently (1). Compared to the peroral peptide administration, higher bioavilabilities and avoidance of liver first pass effect are attractive features. Drug transport and metabolism on the cellular level in nasal epithelia is, however, incompletely understood (2). In vitro cell culture systems are promising tools for the investigation of biological processes and transport phenomena on a cellular level. In this sense, several cell culture models have been used to examine metabolism and transport mechanisms through epithelial barriers. A recent example is the human colon carcinoma cell line Caco-2, which shows transport characteristics of intestinal enterocytes and, therefore, seems to hold some promise for mechanistic studies of drug absorption (3,4).

Cell lines from cancer tissue are easily maintained in culture, but they often have not the morphology or the biochemical characteristics of the original tissue. In contrast, primary cell cultures are difficult to cultivate, but may reflect more closely the original properties. Two commercially available nasal cell lines were examined in this study. The RPMI 2650 cell line originates from a human nasal septum tumor with diploid karyotype (5). Different studies concerning the transcription properties (6,7), the cytokeratin pattern (8) or the metabolic activities (9) have been published. The BT cell line was derived from normal newborn bovine nasal turbinate epithelia. This cell line has mainly been used for viral studies in the past (10–12). The aminopeptidase activities in BT cell homogenates were found to be comparable to those of excised nasal epithelial cells (13). These two cell lines, therefore, seem to be interesting candidates for establishing an in-vitro cell culture model, allowing studies of nasal transport phenomena. Furthermore, human nasal cells in primary culture were examined.

To characterize morphological differences between these cell culture models and the original nasal tissue we applied several methods such as light microscopy, phalloidin staining of cellular actin components and the lectin-binding pattern of the cells. Lectins are glycoproteins with the ability to recognize specific sugar residues. These characteristics make them suit-

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able for various investigations, i.e. for drug targeting (14), for the cancer research (15) or for morphological and histochemical studies on different normal tissues, including intestinal (16), brain (17) and airway (18,19) cell types.

The aims of this investigation were to the following: to examine the FITC-labelled lectin staining pattern of normal human respiratory epithelium, to determine the morphological and histochemical properties of three different nasal cell culture systems; and to assess the suitability of the in-vitro cell culture models for nasal transport studies.

MATERIALS AND METHODS

Paraffin Embedding of Freshy Excised Human Nasal Epithelium

Nasal tissue specimens of 1–3 cm^2 surface area were obtained from patients undergoing turbinectomy. Two pieces were fixed with 4% formaldehyde in phosphate buffered saline (PBS) at room temperatur and dehydrated in a graded series of ethanol. Afterwards they were embedded in paraffin wax, cut into 5 μm thick sections and placed on gelatine-covered microscope slides.

Cell Cultures

Newborn bovine turbinate cells (BT; ATCC CRL 1390) and human septum tumor cells (RPMI 2650; ATCC CCL 30) were obtained from the American Type Culture Collection, Rockville, MD both in passage 24. The cells were cultivated and passaged in polystyrene petri dishes of 10 cm diameter (Nunc, Germany) at 37°C in an atmosphere of 10% CO_2 and 95% relative humidity. The culture medium, which consisted of Dulbecco's modified Eagle's medium (DMEM, Gibco, Germany) supplemented with 4.5 g/L glucose, 10% fetal calf serum (FCS, Biozol, Germany), 1% non-essential amino acids, 1% L-glutamine and 100 U/mL penicillin and 100 μg/L streptomycin (all Gibco, Germany), was changed every second day.

Human nasal epithelial cells in primary culture were cultivated under the same conditions as described previously (20).

Attachment, proliferation and morphology of the cells were observed through an inverse phase contrast microscope (Nikon) with a ×100 magnification.

Lectin Staining

For staining studies the cells were seeded at densities of 10^5 cells/cm^2 on Lab-Tek Chamber slides (Nunc, Germany) and used at days 4, 7, 11, and 18.

Wheat germ agglutinin (WGA), soybean agglutinin (SBA), Dolichos biflorus agglutinin (DBA, all from Sigma, Germany); Peanut agglutinin (PNA) and Concanavalin A (Con A, both from ICN, Germany) were dissolved in PBS, pH 7.2, containing the chloride salts of calcium, magnesium and manganese (0.1 mM of each) to a final concentration of 0.1 mg/mL. The paraffin sections of human nasal tissue were dewaxed in a graded series of ethanol, rehydrated and incubated with 50 μL of the lectin solution for 30 min at room temperatur under light exclusion. Afterwards they were rinsed three times with PBS and examined immediately under a Zeiss fluorescent microscope with an excitatory wavelength of 485 nm and an emission wavelength of 510 nm (BP 485, FT 510 filterset 17, Zeiss, Germany) using a ×312 magnification.

To remove terminal sialic acid residues, possibly blocking the sugar binding sites, parallel sections were pretreated with neuraminidase (Clostridium perfringens, Boehringer Mannheim, Germany) for 24 h at 37°C at a concentration of 0.025 U/mL in 0.2 M citrate-maleate buffer containing 0.9 mM $CaCl_2$. Subsequently they were stained with lectins as described above.

The cultured monolayers of BT, RPMI 2650 or human nasal cells were allowed to equilibrate in PBS at 37°C for 15 min and either directly stained with fluorescence-labelled lectins or fixed prior to lectin labelling using 4% formaldehyde in PBS for 10 min, rinsed and and treated with 1% Triton X-100 (Gibco) on ice for 5 min. After repeated washings with PBS they were also stained using lectins as mentioned above.

Each lectin was checked for its binding specifity by blocking the binding sites with the appropiate sugars (Merck, Darmstadt) prior to the incubation.

Microscopic photographs were taken using a Contac RTS II camera (Germany) and Fuji 100 ASA films.

Actin Staining

F-actin was stained using FITC-labeled phalloidin (Sigma, Germany) as previously described by Anderberg et al. (21). Briefly, 10 μL of the stock solution (200 U/mL phalloidin in methanol) was evaporated and redissolved in 400 μL 0.2 M phosphate buffered saline, pH 7.4 (PBS). The cells were rinsed three times with PBS, fixed for 10 min in 4% formaldehyde in PBS, rinsed again and treated with 1% Triton X-100 (Gibco) on ice for 5 min. After repeated washings and air-drying, the monolayer was stained with FITC-phalloidin under light exclusion for 20 min. The sample was rinsed twice with PBS and examined under a Zeiss fluorescent microscope with an excitatory wavelength of 485 nm and an emission wavelength of 510 nm (BP 485, FT 510 filterset 17, Zeiss, Germany) with a ×500 magnification.

Microscopic photographs were taken with a Contac RTS II camera (Germany) and Fuji 100 ASA films.

Transepithelial Electrical Resistance (TEER)

For TEER measurements confluent cell monolayers, grown for at least 6 days on polyethyleneterephthalate filters (0.4 μm pore size, 4.7 cm^2 area, Becton-Dickinson) were used. The monolayers were allowed to equilibrate in HBSS for 15 min at 37°C, rinsed, placed in the Endohm (WPI) measurement chamber. The basolateral compartment contained 1 mL, the apical compartment 3 ml of fresh HBSS at room temperature. The observed TEER value was corrected for the blank filter resistance.

RESULTS

Lectin Staining of Freshly Excised Human Nasal Tissue

To determine the natural FITC-labelled lectin staining pattern of human nasal tissue in vivo we selected five different lectins, which were used for staining of airway cells before

(18,19). The origin, the carbohydrate specifities and the blocking sugars of the lectins used are listed in Table I.

PNA

PNA was the most effective lectin to label mucus in goblet cells in this study. Neither ciliated nor basal cells nor the lamina propria were stained (Fig. 1a). This staining pattern was not changed by neuraminidase treatment, but the effectivity of the enzyme treatment could be demonstrated by the PNA staining of the desialilated erythrocyte membranes in tissue sections.

WGA

This lectin stained extensively the goblet cell mucus. Moreover, the lamina propria showed an unspecific labelling and the cilia were weakly stained (Fig. 1b). Neuraminidase digestion did not alter the lectin binding pattern.

SBA

Just as WGA and PNA this lectin labelled the mucus in goblet cells, but in contrast the staining was punctiform. Only some areas of the goblet cells were stained, indicating the complex composition of the mucus in the cells (Fig. 1c). With neuraminidase digestion the lectin-binding pattern was not altered.

DBA

DBA did not stain the epithelium or the laminar propria at all (Fig. 1d). Neuroaminidase treatment could not improve this staining.

Con A

Con A labelled the lamina propria of the respiratory epithelium and the apical cytoplasm of the ciliated cells. Furthermore the cell membranes were stained. The mucus in the goblet cells was not stained and appeared as black holes (Fig. 1e). After neuraminidase digestion the mucus within the goblet cells was slightly stained (Fig. 1f).

For further cell culture characterization we select Con A to determine the epithelial origin of the cultured cells, PNA as a marker for goblet cells and WGA to recognize ciliated cells.

Table I. Lectin Origin Including Sugar Specifities and Inhibitory Saccharides

Lectin	Agglutinin Origin	Carbohydrate Specifity	Inhibitor
PNA	Arachis hypogaea	β-D-Gal-D-GalNAc β-D-GalNAc, Gal	Galactose
WGA	Triticum vulgare	GlcNAc, NANA[1]	N-acetylglucosamine
Con A	Canavalia ensiformis	α-Man, α-Glc	Mannose
SBA	Glycine max	α-D-GalNAc β-D-GalNAc, Gal	N-acetylgalactosamine
DBA	Dolichos biflorus	α-D-GalNAc, Gal	N-acetylgalactosamine

[1]N-acetylneuraminic acid.

CELL CULTURES

Light Microscopy

Isolated human nasal cells in primary culture grew to a confluent monolayer after 6–8 days in culture (Fig. 2a), as previously described (20).

BT cells were spindle-like and spread from clusters to an optically tight monolayer in 5–7 days when seeded at densities of 10^5 cells/cm^2 (Fig. 2b). With increasing time and cell number cell clusters disappeared and a homogenous growth could be observed (not shown)

In contrast, RPMI 2650 cells showed a small and round shape. After seeding they form colonies (Fig. 2c) and grew to multilayered cell aggregates. These aggregates did not form a monolayer even after 3 weeks in culture neither on plastic dishes nor on Falcon filters.

Lectin Staining Pattern of Cultured Cells

After 4, 7, 11, and 18 days in culture the surface lectin-staining pattern from all three cell culture systems was determined. None of these staining pattern changed with increasing culture duration. To enable the staining of intracellular structures like cell nuclei or mucus granules we fixed the monolayer and perforated the plasmamembrane by Triton-X 100 treatment.

PNA

PNA showed a slight labelling of the plasmamembrane of BT cells, independent of the pretreatment. Mucus or goblet cells, which should have been labelled by PNA were not detected (Fig. 3a).

RPMI 2650 cells also showed no difference between fixed and unfixed monolayers. The plasmamembranes revealed moderate binding sites for PNA (Fig. 3b).

As demonstrated in Figure 3c, the labelling of unfixed human nasal epithelial cells in primary culture demonstrated a distinct pattern. The plasmamembrane again was slightly stained but in addition either entire cells or the central area of the cells appeared fluorescent while others were not labelled, suggesting the presence of goblet cells within the monolayer.

By treating a fixed monolayer of human nasal epithelial cells with PNA this staining pattern changed. The staining of goblet cells disappeared, indicating a wash-out effect of the mucus (not shown).

WGA

The fluorescence intensity of WGA was stronger than PNA, but again unfixed monolayers of BT (Fig. 4a) and RPMI 2650 (Fig. 4b) cells revealed only a slight homogenous staining of the plasmamembrane. Bright fluorescent dots on the monolayers were detached, probably dead cells filled with lectines. After fixation and perforation of the monolayer the cell nuclei were stained in both cell types, but goblet cells were not present.

Human nasal cells in primary culture showed beneath the staining of the plasmamembrane a higher fluorescence of some cells and especially of the cilia (Fig. 4c). By fixing the monolayer, the fluorescence of the cilia disappeared and in agreement with the other cell lines the cell nuclei became labelled (not shown)

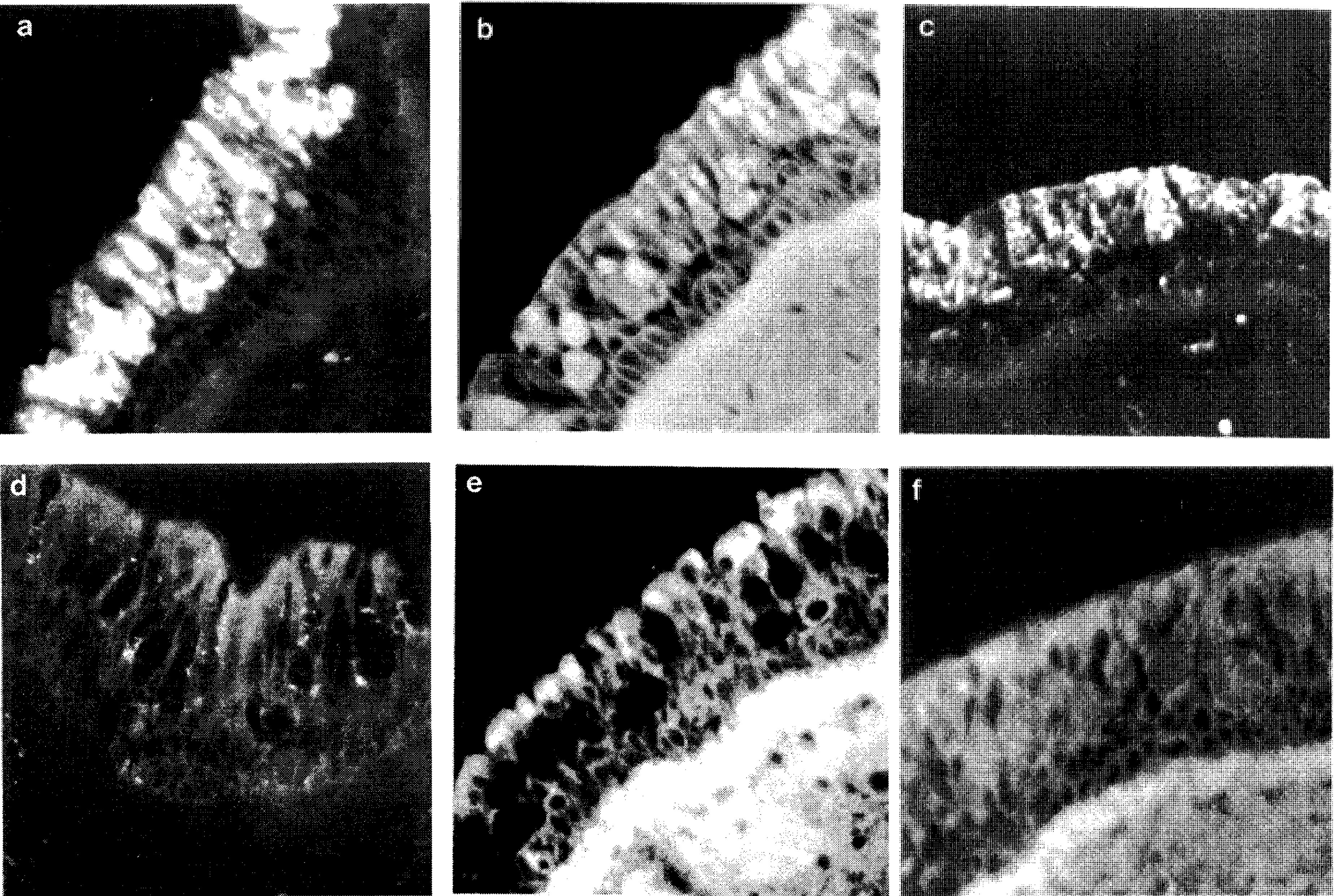

Fig. 1. Lectin staining pattern of paraffin embedded sections of human nasal epithelium. a) PNA labelled the mucus in goblet cells. b) WGA showed also an intensive staining of the mucus and a slight affinity to the cilia. c) SBA labelled the mucus in a punctiform way. d) DBA showed no affinity to the human respiratory epithelium. e) Con A stained the apical cytoplasm and the lamina propria. f) After neuroaminidase treatment a slight staining of the mucus by Con A was noticed. (500x) (Original reproduced at 89%).

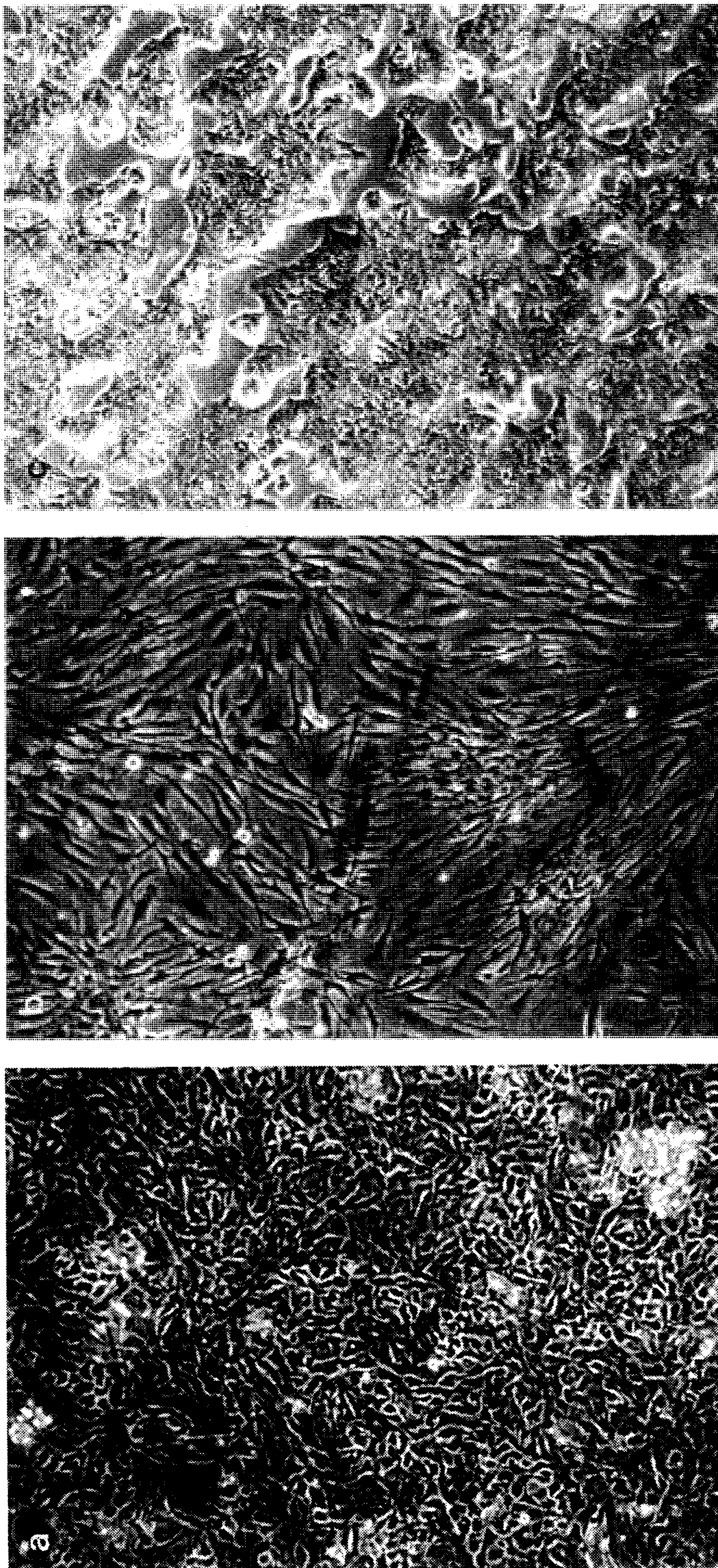

Fig. 2. Growth and shape of different nasal cell cultures. a) Human nasal cells in primary culture grew to a confluent monolayer 8 days after seeding. b) BT cells showed a spindlelike shape and formed a monolayer 3 days after seeding. c) RPMI 2650 cells grew to a nonconfluent multilayer consisting of small cells 5 days after seeding. (100x) (Original reproduced at 89%).

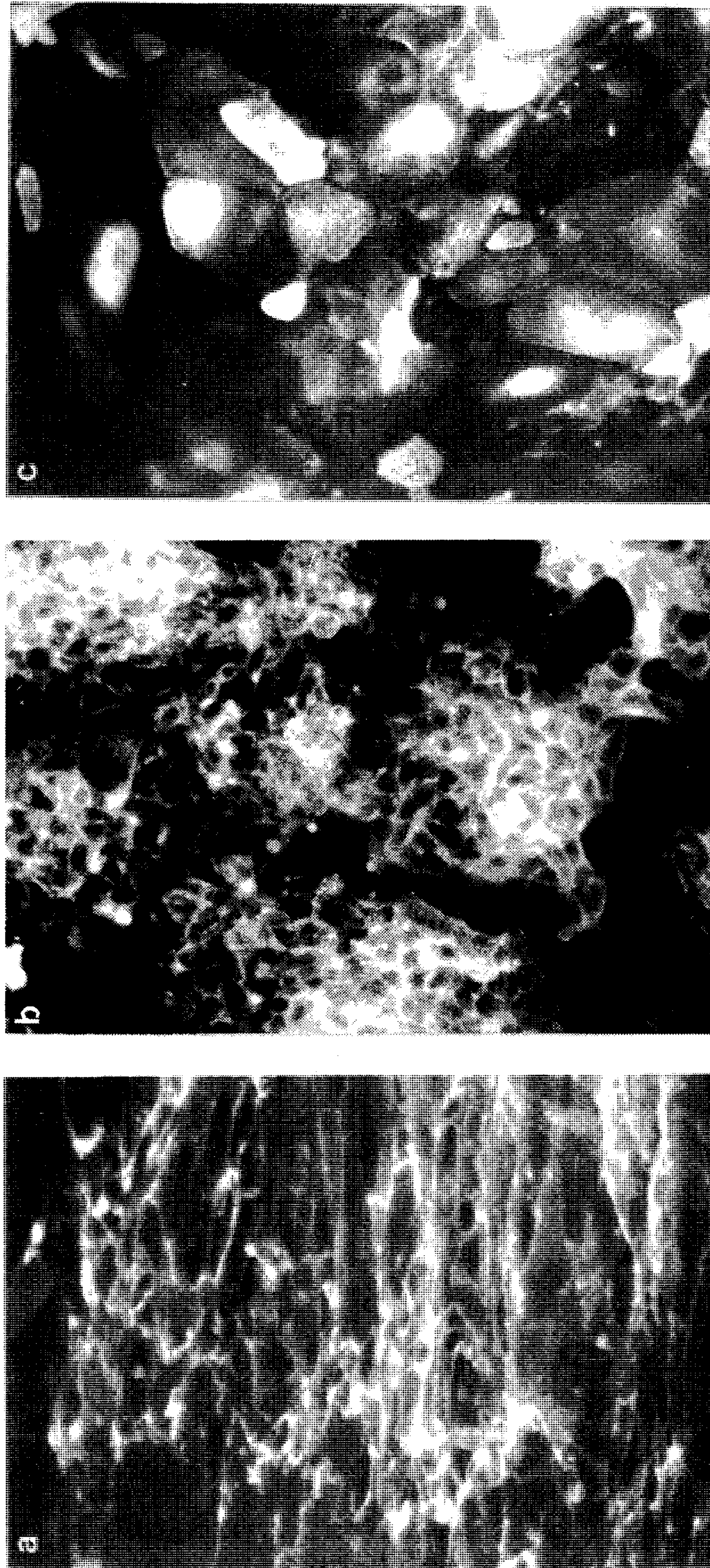

Fig. 3. FITC-coupled PNA staining of nasal cell cultures to demonstrate goblet cell expression. a) Neither BT cells nor b) RPMI 2650 cells expressed goblet cells. c) In contrast human nasal cells in primary culture showed fluorescent cells, indicative for goblet cells (312x) (Original reproduced at 83%).

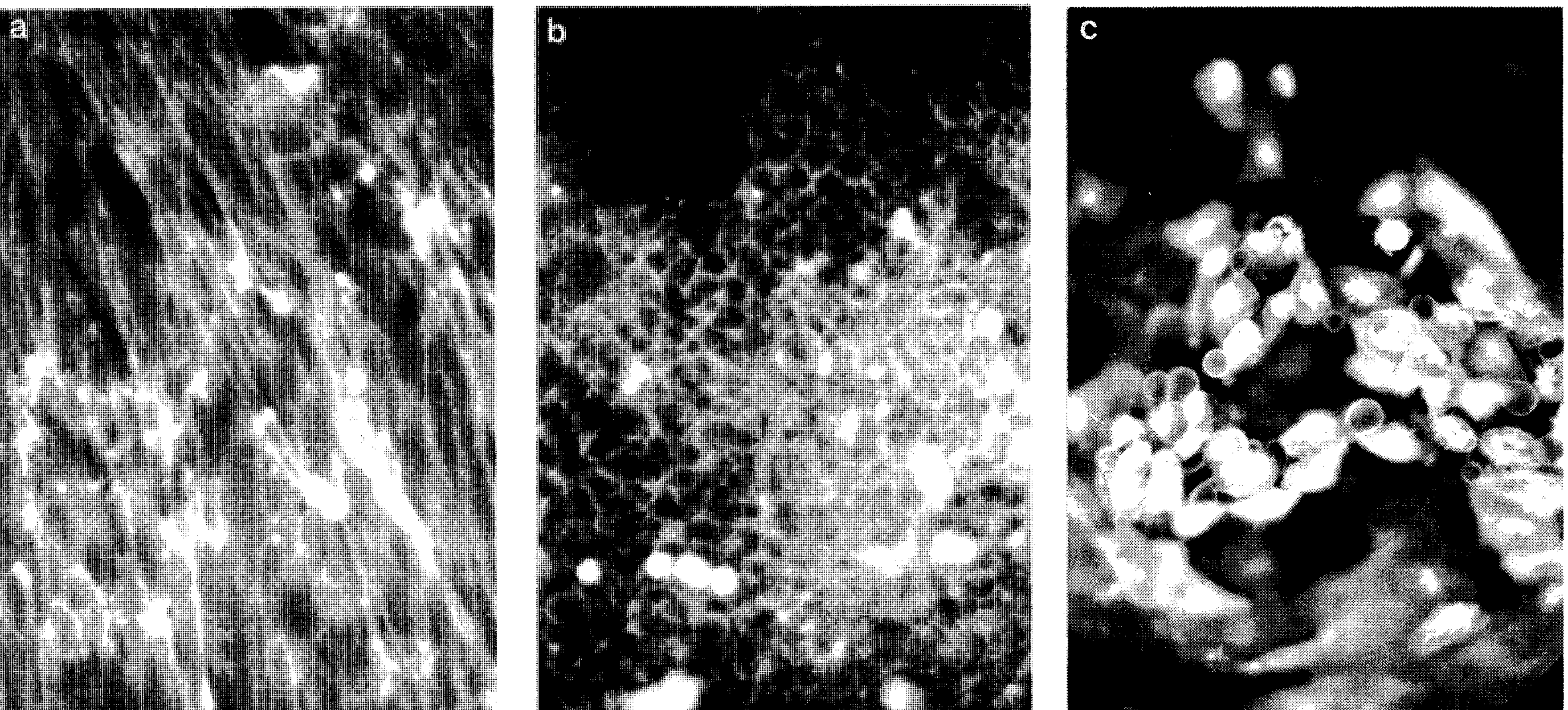

Fig. 4. FITC-coupled WGA staining of nasal cell cultures to demonstrate ciliated cells. a) Neither BT cells nor b) RPMI 2650 cells expressed cilia. c) In human nasal cells in primary culture cilia were intensively stained by WGA (312x) (Original reproduced at 83%).

Con A

RPMI 2650 cells were not stained by Con A in contrast to BT monolayers which revealed a staining of the nucleus surrounding area after but not prior to fixation (Fig. 5a).

Fig. 5b showed a labelling of some cell nuclei within the monolayer of primary cultures. This fluorescence decreased when the monolayers were treated with formaldehyde and Triton-X 100.

Actin Staining and TEER Measurement

Apart from the expression of different types of epithelial cells another sign of a functional nasal epithelium is the regular development of the perijunctional cytoskeleton associated with tight junctions (22). This cytoskeleton could be detected by actin fibre staining using FITC-phalloidin allowing its visualization by fluorescence microscopy. The development of tight junctions was also verified by measurement of the transepithelial electrical resistance (TEER)

BT cells formed an optically tight monolayer but the actin staining presented a network of actin fibres, as demonstrated in Fig. 6a. This could be the terminal web or stress fibres, expressed by cultured cells to allow cell adhesion. However, a clear demarcation of the cells characterized by a belt-like FITC-phalloidin staining was not observed after 4, 7, 11, or 18 days in culture, indicating the failure of formation of tight junctions. These findings were confirmed by TEER values in the range of the blank filters controls even after 21 days in culture.

As expected, RPMI 2650 cells showed no increase of TEER with time since a homogeneous cell monolayer was not formed. Surprisingly the FITC-phalloidin staining of the cell aggregates was positiv from day 4 to the end of observation period at day 18 (Fig. 6b).

Human nasal epithelial cells in primary culture grew to a confluent monolayer in 6–8 days. At this time a well-organized perijunctional cytoskeleton was formed, as revealed in Fig. 6c. When seeded on polyethyleneterephthalat filters TEER values increased from 357 ± 68 ohm*cm^2 at day 6 to 638 ± 99 ohm*cm^2 at day 9. These values are in the range of TEERs measured in other comparable epithelial cell lines like Caco-2 (4).

DISCUSSION

FITC-labelled Lectin Staining Pattern of Human Respiratory Tissue

Lectins were often used in the literature to determine the histochemical properties of various epithelia (23–30). Obviously the lectin-staining patterns are species-, tissue- and differentiation-dependent (Table II). Furthermore, the choice of the fluorescent label and the fixation conditions are known to influ-

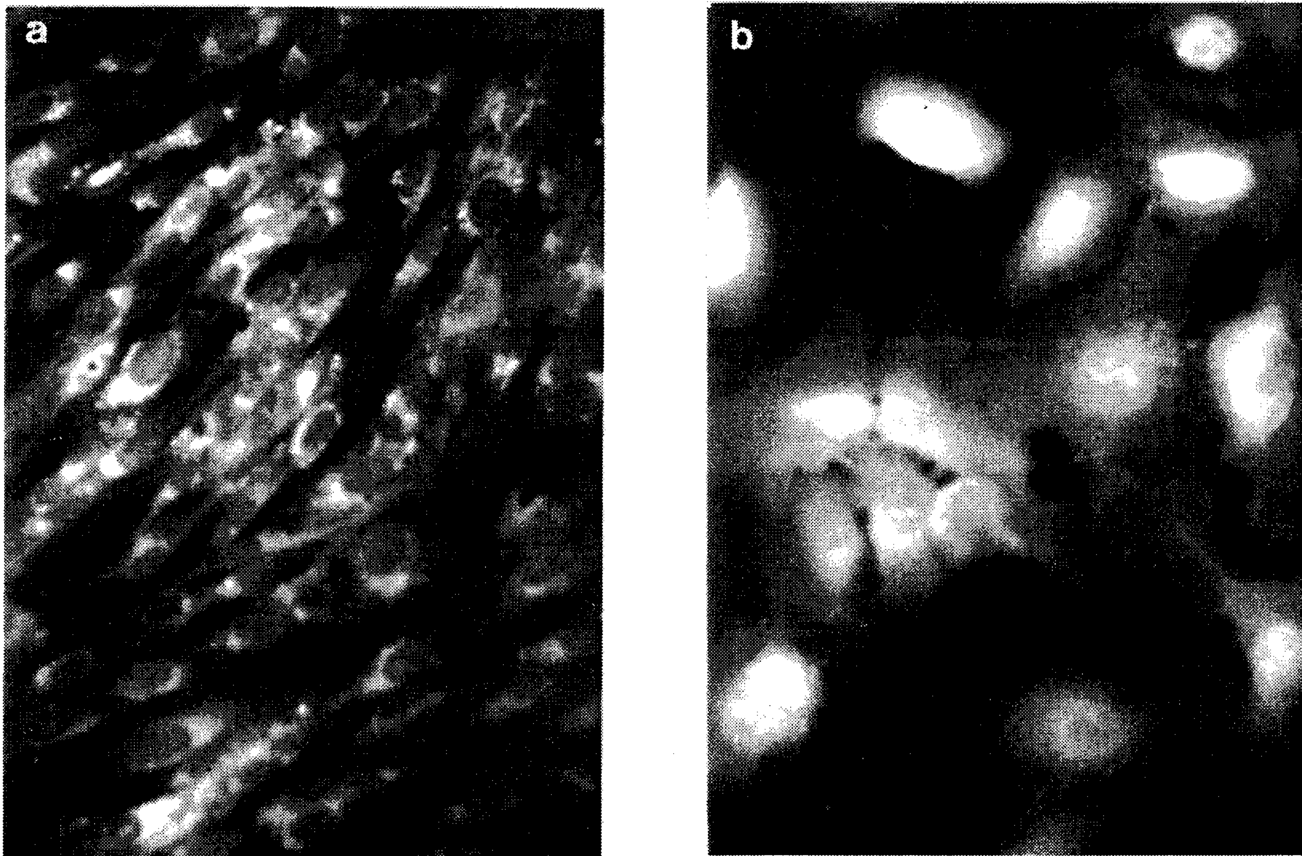

Fig. 5 a) Con A labelled the nuclei surrounding area of BT after fixation. b) Human nasal epithelial cell monolayers showed fluorescent cell nuclei after staining with Con A (312x).

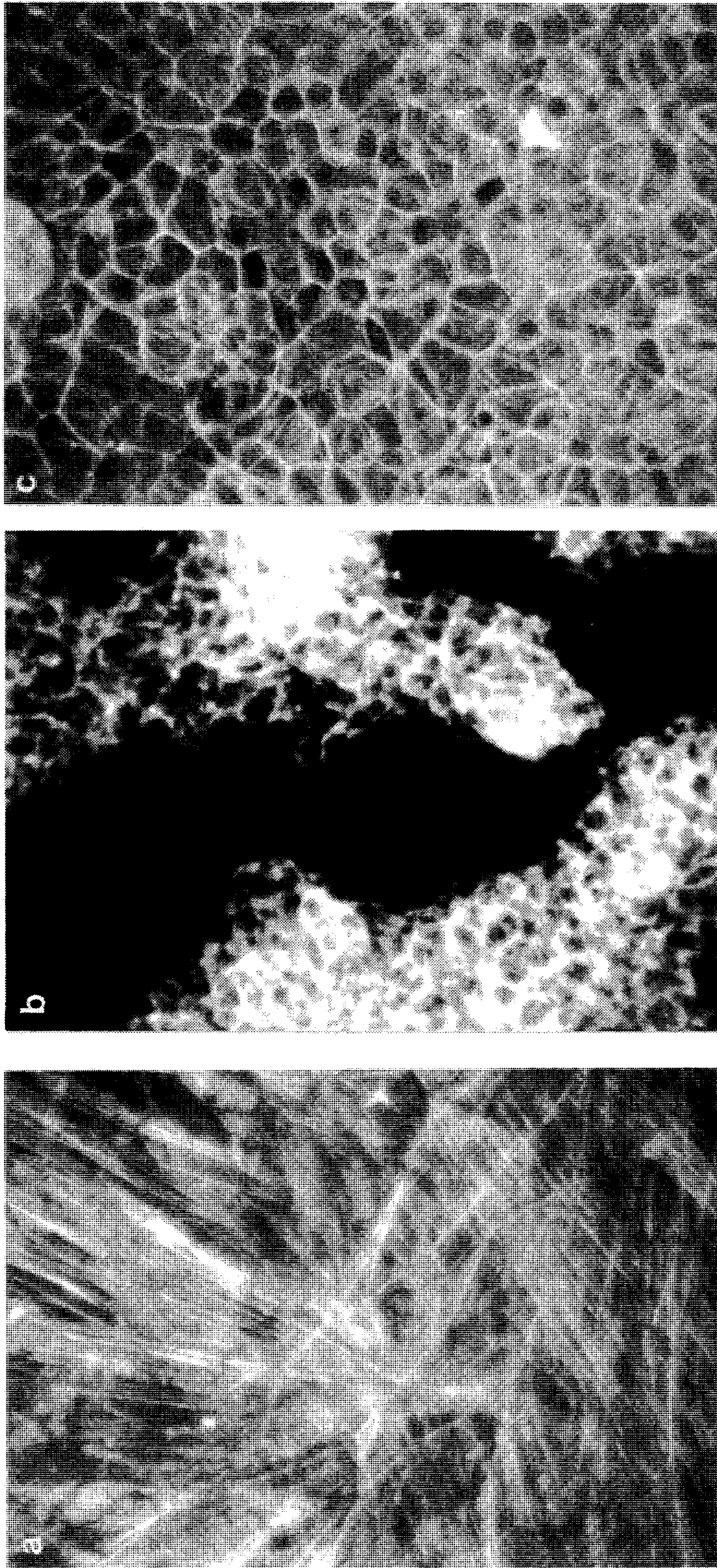

Fig. 6. Phalloidin staining of intracellular actin fibres to demonstrate the perijunctional cytoskeleton. a) BT cells did not express the junctional complex but stress fibres to allow cell attachment. b) RPMI 2650 cells showed perijunctional actin rings but did not form a confluent monolayer. c) Human nasal cells in primary culture showed the junctional complex as well as a confluent monlayer (312x) (Original reproduced at 89%).

Table II. Lectin Staining Pattern of Human Nasal Epithelium Compared with Data from Other Species Known from the Literature, Regarding Only the 5 Lectins Used in this Study

	Human Nose (FITC)	Human Nose[1] (HRP)	Human Bronchus[2]	Mouse Nose[3]	Sheep Trachea[4]
Cytoplasm	Con A	Con A WGA	—	Con A	—
Goblet Cells	PNA WGA SBA	PNA WGA	PNA WGA SBA	PNA SBA	DBA SBA
Cilia	WGA	—	—	WGA	DBA SBA

Data obtained from
[1]Gulisano et al. (23).
[2]Mazzuca et al. (24).
[3]Lundh et al. (26).
[4]Mariassy et al. (27).

ence the lectin-binding (30). Gulisano et al. (23) examined the carbohydrate composition of the human nasal epithelium with horseradish-peroxidase-conjugated (HRP) lectins. Our studies with FITC-labelled lectins revealed a slightly different pattern, e.g. WGA failed to stain the cytoplasm in our study (for comparison see Table II) probably due to different fixation procedures and the altered binding specifity of FITC-coupled lectins.

Morphological and Histochemical Properties of Nasal Cell Cultures

In our study with human nasal epithelial cells, PNA was a marker for mucus in goblet cells, WGA labelled mucus and cilia and Con A stained the cytoplasm. For this reason, these three lectins were used to determine the differentation status of the nasal cell lines and the primary culture of human nasal epithelial cells.

BT is an animal nasal cell line derived from bovine turbinate of newborn calves. Since it is a non-tumorogenic cell line, the cells probably retained their original characteristics and could be used for nasal transport and metabolism studies. Audus and Tavakoli-Saberi (13) determined recently the morphology and the enzyme activity of BT cells. They described the formation of a monolayer after 7–10 days in culture and a cuboidal shape of the cells. Furthermore, they observed the presence of domes, characteristic for ion transport and tight junctions, occasional microvilli and granules in the monolayer. We could not confirm these findings. In our laboratory BT cells grew to monolayers, but they exhibited a spindle-like shape and domes were not observed. In addition, a concrete perijunctional actin ring was not developed over the culture time of 18 days and a TEER was not measurable.

Lectin staining did not reveal any granules in the bovine monolayer, which should have been stained by WGA or PNA. As peviously discussed, the carbohydrate composition in mucus of airway cells is species dependent. The failure of lectin binding to bovine cell granules could be due to different sugar moieties in the mucus, although it is unlikely that neither galactose nor N-acetylglucosamine nor mannose residues exist in bovine mucus. The differences between Audus and our results are possibly due to different culture conditions, since they used equine serum instead of fetal calf serum which could promote the expression of different cell types.

The plasmamembrane of BT cells revealed a few N-acetylglucosamine or N-acetylneuraminic acid and galactose—residues, as demonstrated by the weak labelling with WGA and PNA. In addition, the cell nucleus was covered with N-acetylglucosamine or N-acetylneuraminic acid moieties whereas the perinuclear region was stained by Con A, indicating mannose residues present on the endoplasmic reticulum. This staining pattern was reported for cultured fibroblasts, with a higher affinity for mannose-specific lectins to bind to the endoplasmic reticulum whereas WGA bind exclusively to the Golgi complex (30).

RPMI 2650 are human nasal cells originating from a spontaneously formed tumor. Little is known about the morphology of these cells. As reported previously (5) the cells were very small and grew densely in cell "clumps". This could be confirmed by our results, where the cells firstly formed small colonies and then grew to multilayered cell aggregates. The cells were much smaller than BT cells and appeared in shape more similar to human epithelial cells. The characterization with lectins demonstrated, that neither goblet cells, determined by PNA, nor ciliated cells (WGA) developed over the cultivation time, indicating the low differentation of the cultured cells. This low degree of differentation and consequently the changed lectin binding pattern is a common phenomenon of cancer cells (30).

We observed the formation of the perijunctional rings within the cell clusters but a transepithelial electrical resistance was not developed, since a confluent monolayer was not formed.

Consequently, RPMI 2650 cells have not been used for transport studies so far. Recently Peter et al. (9) determined the aminopeptidase activity of these cells, demonstrating at least in this regard some potential use of RPMI 2650 cells for nasal metabolism studies.

Human nasal cells in primary culture grew within 6–8 days to a confluent monolayer. Actively beating cilia, observed through the inverse phase contrast microscope, demonstrated the high differentiation of the cultured cells. This observation could be confirmed by lectin staining. PNA as well as WGA, both indicative for goblet cells, stained some cells within the monolayer. Furthermore WGA labelled intensively the still beating cilia of the unfixed cell culture.

As discussed above, the cilia of paraffin embedded sections of human respiratory epithelium showed only a weak staining of cilia by WGA. This could be caused by the embedding and fixation procedure (30), since formaldehyde influenced the structure of cell proteins. Accordingly, fixation of cultured nasal cells resulted in the failure of WGA binding to the cilia.

Further evidence for the well differentiated human nasal cell culture was the development of the cytoskeleton and tight junctions, as revealed by actin staining and TEER measurements.

Suitability for Transport Studies

To conduct transport studies through epithelial cell monolayers it is necessary, that tight junctions are developed. Furthermore, the cells should be in a high differentiation state, similar to the in-vivo situation, to allow the transfer of the obtained results to animal data.

These requirements were fulfilled by human nasal cells in primary culture. These cells were highly differentiated and formed tight junctions. BT cells grew to an optically tight monolayer but did not develop tight junctions. RPMI 2650 cells grew multilayered and did not form a confluent monolayer, although they develop perijunctional actin rings. For this reason, transport studies were not conducted with RPMI 2650 cells.

In summary, excised human nasal epithelium revealed binding sites for WGA, PNA, SBA and Con A. Mucus in goblet cells could be stained by PNA, WGA and SBA, whereas cilia were labelled by WGA. Con A showed only a moderate affinity to the apical cytoplasm of the cells. According to these observations we selected PNA, characteristic for goblet cells, WGA to label cilia and Con A to determine the epithelial origin of the cells for histochemical evaluation of different nasal cell cultures.

In combination with light microscopy, actin staining and TEER measurement, lectins were useful tools to determine the origin, differentiation state and the suitability for transport studies of the various cell cultures. BT and RPMI 2650 cell were found to be undifferentiated and not suitable for transport studies. In contrast, human nasal cells in primary culture showed goblet and ciliary cells and developed tight junctions, necessary for transport experiments. Our data suggest that human nasal epithelial cells in primary culture seem to be a cell culture model which holds some promise for nasal transport and metabolism studies.

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