Characterisation of lectin binding patterns of mouse bronchiolar and rat alveolar epithelial cells in culture

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Summary

Lung epithelial cell differentiation pathways remain unclear. This is due in part to the plasticity of these cells and the lack of markers which accurately reflect their differentiation status. The aim of this study was to determine if lectin binding properties are useful determinants of functional differentiation status *in vitro*. Mouse Clara cells were cultured for 5 days. During this time, no alteration in differentiation was evident by electron microscopy. No significant alteration in binding reactivity of *Bauhinia purpurea* (BPA), *Maclura pomifera* (MPA), Concanavalin A, Wheat germ or Helix pomatia lectins occurred in cultures compared with Clara cells in mouse lung tissue. In contrast, nitrotetrazolium blue reductase activity and CC10 expression declined in culture. Rat type II cells were cultured for 8 days. Between days 0 and 4, the number of type II cells identified by electron microscopy was constant at 70–80%, decreasing to 8% by day 6. In contrast, by day 4, only 42% cells retained alkaline phosphatase activity. BPA and MPA reactivity was altered at day 0 and day 4 respectively, compared with cells *in situ*. Therefore, the reactivity of lectins analysed here does not reflect functional differentiation status of cultured mouse Clara cells. However, BPA and MPA reactivity may be a sensitive indicator of alterations in rat type II cell differentiation *in vitro*.

Introduction

The bronchiolar and alveolar regions of the lung are lined by a single layer of epithelial cells. At least 8 subpopulations have been described within this epithelium, each with important, and often overlapping roles in lung function. For example, bronchiolar Clara cells and alveolar type II cells store and secrete components of the protective mucous and surfactant layers respectively. They contain high levels of biotransforming enzymes involved in detoxification, but also activation, of certain compounds (Myles *et al.* 1989, Kudo 1994, Quondamatteo *et al.* 1998). In addition, they both serve as progenitor cells in their respective regions (Massaro 1989, Voelker & Mason 1989). Methods for isolating relatively pure populations of Clara and type II cells have been described for several species, including human, providing excellent opportunities to study specific cell functions described above. However, the propensity of both of these cell types for rapid differentiation and loss of characteristic features once placed in culture has hindered full exploitation of these model systems. In addition, the identities of the cell types which subsequently arise in culture are, as yet, unclear. A lack of cell specific markers along with the ability of certain

lung epithelial cell types apparently to differentiate, dedifferentiate and redifferentiate under various conditions (Johnson *et al.* 1990), has meant that cultures of such cells are poorly defined. Consequently, studies have mainly focused on *in vivo* animal experiments and more stable transformed cell lines.

Lectins have been used as tools to distinguish between lung cell types on the basis of their binding to specific carbohydrate groups, usually at the cell surface (Geleff *et al.* 1986, Tatrai *et al.* 1994). These lectin binding sites may be involved in intracellular recognition (Sato & Muramatsu 1985) and binding of yeast, bacteria and other microorganisms to host cells (Mouricout 1997). For example, *Maclura pomifera* agglutinin (MPA) binds to galactose (α-D-Gal) or N-acetylgalactosamine (α-D-GalNAc) residues while Concanavalin A (ConA) binds to glucose (α-D-Glc) and mannose (α-D-Man) residues. The specific nature of these interactions means that lectins are potentially useful markers for early epithelial changes in lung disease (Kasper *et al.* 1994) and ontological studies (Joyce-Brady & Brody 1990) as well as markers for *in vitro* differentiation investigations. However, as yet, few such *in vitro* studies have been carried out. Furthermore, many of the reports of lectin binding to either lung tissue or cultured cells published so far are contradictory.

For example, Brandt (1982) reported that ConA bound to type II cells in rat lung tissue while Dixon and Jersild (1983) and Williams (1984) found little to no binding of ConA to these cells. Similarly, Brandt (1982) found that *Ricinus communis* (RCA) bound to type I but not type II cells in rat lung tissue while Taatjes *et al.* (1990) observed RCA binding to both type I and type II cells in rat lung. In addition, in cultured rat type II cells, Dobbs *et al.* (1985) reported that MPA was bound progressively less and RCA bound more over time in culture while Kovacikova *et al.* (1999) observed no change in the levels of MPA or RCA binding to these cells in culture. Interspecies variations are also apparent with *Bauhinia purpurea* agglutinin (BPA) reportedly binding to both type I and type II cells in human lung (Sarker *et al.* 1994) but only to type I cells in rat lung tissue (Kasper *et al.* 1994).

In an attempt to clarify this contentious area and to assess the potential of lectins as markers in primary lung cell culture studies, we have examined the binding of a panel of lectins to rat lung tissue as well as cultured rat type II cells in conjunction with measurements of alkaline phosphatase acitivity as an indicator of cell function, and ultrastructural characterisation by electron microscopy (EM). We have also carried out the first in-depth study of lectin binding to cultured mouse Clara cells, again in parallel with analyses of other Clara cell biochemical markers and EM to further characterise the phenotypes of mouse Clara cells and their derivatives in culture.

Materials and methods

Animals

Mice used were C57/Black 6, bred in-house and weighed on average 35 g. Male Sprague–Dawley rats (Charles River, Hungary, Isaszeg) weighing 190–210 g were used after 1 week quarantine in our animal house (accredited according to GLP). The animals were fed with the standard chow diet from the same supplier.

Isolation and culture of mouse Clara cells

Mice were sacrificed by intraperitoneal injection of pentabarbitone. Lungs were perfused with saline, digested with trypsin and Clara cells were isolated as described previously (Masek & Richards 1990). The procedure used here differed from that of Masek and Richards (1990) as follows: gentamycin and anti-PPLO were omitted from all solutions and the medium used throughout the isolation and for culture was a 1:1 mixture of Hams F12 (Gibco) and M-199 medium (Gibco) supplemented with 2 mM L-glutamine, 10 µg/ml insulin, 5 µg/ml transferrin, 100 ng/ml hydrocortisone, 10 ng/ml EGF, 0.1 ng/ml retinyl acetate and Pen/Strep (Gibco, 100 U/ml penicillin, 100 µg/ml streptomycin). Once isolated, cells were either cytospun onto glass slides (day 0) or plated onto 16-well glass chamber slides (Gibco) which had been pre-coated with 50 µg/ml fibronectin, and incubated at 37 °C, 5% CO_2. Cells were allowed to attach overnight after which the medium was replaced to remove dead and unattached cells. Medium was subsequently replaced every 2 days.

Isolation and culture of rat type II cells

Rats were deeply anaesthetised with 60 mg/kg pentobarbitone (Nembutal, Sanofi, Paris, France) intraperitoneally, and then killed by severing the abdominal aorta. Type II cells were isolated according to methods described previously (Richards *et al.* 1987, Hoet *et al.* 1994). Cells were cytospun onto glass slides (day 0) or plated onto 24-well plates (Falcon) and incubated at 37 °C, 5% CO_2. Culture medium was DMEM (Sigma) supplemented with 10% FCS (Gibco) and Pen/Strep (Sigma; 100 U/ml penicillin, 100 µg/ml streptomycin). Medium was replaced every 2 days.

Lectin histochemistry

For lectin binding to cultured cells, cytospun cells and plated cells were fixed at appropriate time points by incubating at room temperature in 4% buffered neutral formalin (pH 7.4) for 10 s. The following biotinylated lectin agglutinins (Sigma) were used: *Bauhinia purpurea* (BPA, specificity: β-Gal(1→3)GalNAc), Concanavalin A (ConA, specificity: α-D-Man, α-D-Glc), *Helix pomatia* (HPA, specificity: α-D-GalNAc), *Maclura pomifera* (MPA, specificity: α-D-Gal, α-D-GalNAc), Wheat germ (WGA, specificity: (D-GlcNAc)$_2$, NeuNAc). Lectins were diluted to a final concentration of 20 µg/ml in Tris-buffered saline (TBS), pH 7.4, which contained 10 mM $CaCl_2$, 0.2 mM $MgCl_2$ and 1 mM $MnCl_2$. All incubations were carried out at room temperature. Cells were incubated with lectins for 20 min, rinsed with TBS and incubated for 30 min with either avidin–biotin-alkaline phosphatase (AB–AP) or avidin–biotin–horseradish peroxidase (AB–HRP) (Vector Laboratories). Binding was visualised by staining for 10–15 min with Vector Red or 3,3'-diaminobenzidine (DAB) which are substrates for AP and HRP respectively. As controls for specificity, lectins were pre-incubated overnight at 4 °C with appropriate hapten sugars before incubation with cells (BPA: NAcGal; MPA: D-Gal; HPA: NAcGal; WGA: NAcGlc; ConA: Man + Glc).

For lectin binding to tissue, lungs were fixed in 10% buffered neutral formalin (pH 7.4) for 2 days before routine processing and embedding in Paraplast (Sigma). Sections were cut at 4–5 µm, dewaxed, rehydrated and lectin binding was carried out and visualised as described above.

Immunocytochemistry

For CC10 and keratin immunocytochemistry, cells were fixed respectively in formalin as above and in methanol for 7 min at −20 °C. Cells were incubated with primary antibody for 2 h, rinsed with TBS–0.1% Tween 20, incubated with biotinylated secondary antibody for 30 min, rinsed and binding was visualised as above with AB–AP and Vector Red or Vector Blue substrate. For CC10 binding to lung tissue, tissue sections

were obtained as described above, dewaxed, rehydrated and CC10 immunohistochemistry was carried out as described above for cells. The CC10 antibody was a generous gift from Dr. Gurmukh Singh, Deptartment of Veterans Affairs Medical Center, Pittsburgh, Pennsylvania, USA. The keratin antibody (clone MNF116), a pan-keratin antibody which recognised keratins 5, 6, 8, 17 and 19, was bought from DAKO, UK.

Nitrotetrazolium blue assay

The nitrotetrazolium blue (NBT) solution consisted of 100 μl NBT stock solution (Boehringer Mannheim) in 10 ml 0.1 M Tris pH 9.5, 0.05 M $MgCl_2$, 0.1 M NaCl, 0.1% NADPH. Cells were fixed in formalin as above and incubated with the NBT solution for 10 min at 37 °C. Positive cells stained strongly purple.

Alkaline phosphatase

One tablet of Naphthol AS-TR phosphate (Sigma) was dissolved in 10 ml deionised water and 3 tablets of Fast Red RC (Sigma) were dissolved in 5 ml Tris-buffered saline (pH 8.4–8.6). Cells were incubated in a mixture of 5 ml AS-TR and 5 ml Fast Red for 30 min at room temperature. Positive cells were stained bright red. Red cells with 4 or more lamellar bodies were considered to be type II cells.

Electron microscopy

Day 0 cells were pelleted by centrifugation and cultured cells were harvested by trypsinisation and pelleted. Pellets were fixed in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) overnight and post-fixed in buffered 1% osmium tetroxide (0.1 M with respect to sodium cacodylate, and 0.04 M with respect to potassium ferrocyanide) for 2 h. After thorough washing in distilled water, the pellets were stained *en bloc* in 5% aqueous uranyl acetate overnight at 4 °C, followed by dehydration through an alcohol series, and embedding followed by polymerisation in Araldite resin (TAAB Laboratories Ltd., Aldermaston, UK). Ultrathin sections of approximately 90 nm thickness were cut into 300-mesh copper grids, and stained with lead citrate. The sections were examined in a Jeol 100 CX-II transmission electron microscope at an accelerating voltage of 80 kV.

Results

Mouse Clara cells

Lectin binding – lung tissue

BPA, MPA, ConA and WGA reacted strongly with the apical membranes of bronchiolar Clara cells in mouse lung tissue. HPA showed no reactivity. BPA and MPA reactivities are illustrated in Figure 1A and D. Lectin binding was completely blocked or significantly weakened by control hapten treatment.

Lectin binding – cultured cells

The binding activities of the above lectins to mouse Clara cells were examined over 5 days in culture (Table 1). By day 7, cultures had begun to visibly appear necrotic, therefore day 5 was used as the final time point. ConA and WGA both showed very strong binding activity to freshly isolated cells, and all cells were stained. Staining remained strong at day 1 and day 5 in culture. BPA showed no reactivity with freshly isolated cells (Figure 1B) and only a few scattered positive cells (less than 5%) were present at day 1. However, at day 5, 30–40% cells bound BPA (Figure 1C). MPA produced moderately positive staining in freshly isolated cells (Figure 1E) and cultured cells at day 1 and day 5 (Figure 1F) were all strongly positive. HPA showed no reactivity with isolated or cultured cells at any stage.

Electron microscopy

When examined by EM, fresh isolates were found to consist of approximately 78% Clara cells, indicating relatively high levels of purity. The remainder were almost all ciliated cells. Day 1 and day 5 cultures consisted of approximately 80% and 85% Clara cells respectively and again, the remaining cells were almost exclusively ciliated cells. Clara cell viability was high at each time point with less than 25% cells appearing apoptotic or necrotic. However, at day 5, there appeared to be a drop in the mitochondrial size and number per cell compared to day 1.

CC10

CC10 is a protein primarily expressed by bronchiolar Clara cells in rodents and humans (Singh & Katyal 1997). Its function is unclear but it may play a role in regulation of inflammation. CC10 immunoreactivity was demonstrated in the apical membrane regions of bronchiolar cells in mouse lung tissue (Figure 1G). Freshly isolated cells were strongly positive for CC10 immunoreactivity and remained so at day 1 (Figure 1H). Staining appeared stronger within clumps of cells which had not spread fully compared with larger, flattened cells. However, this could be because the unspread cells had a less visible volume than those that had spread, but had an equivalent amount of stain. By day 5 in culture, CC10 immunoreactivity appeared slightly reduced (Figure 1J).

NBT assay

Functional Clara cells contain high levels of NADPH-dependent reductase activity. This can be detected histochemically using an NBT assay in which a colourless NBT solution is converted to a purple formazan product by NADPH-dependent reductase. Brief fixation with formalin prior to staining eliminates the low levels of enzyme activity in other cell types likely to be present. Following staining, approximately 75% freshly isolated cells were dark purple in colour, indicating that these were functional Clara cells (Figure 1J). Most positive cells were present within clumps. The number of NBT-positive cells declined in culture. At day 1 and day 5 (Figure 1K), approximately 50% and 40% respectively stained dark purple. Again, most positive cells were
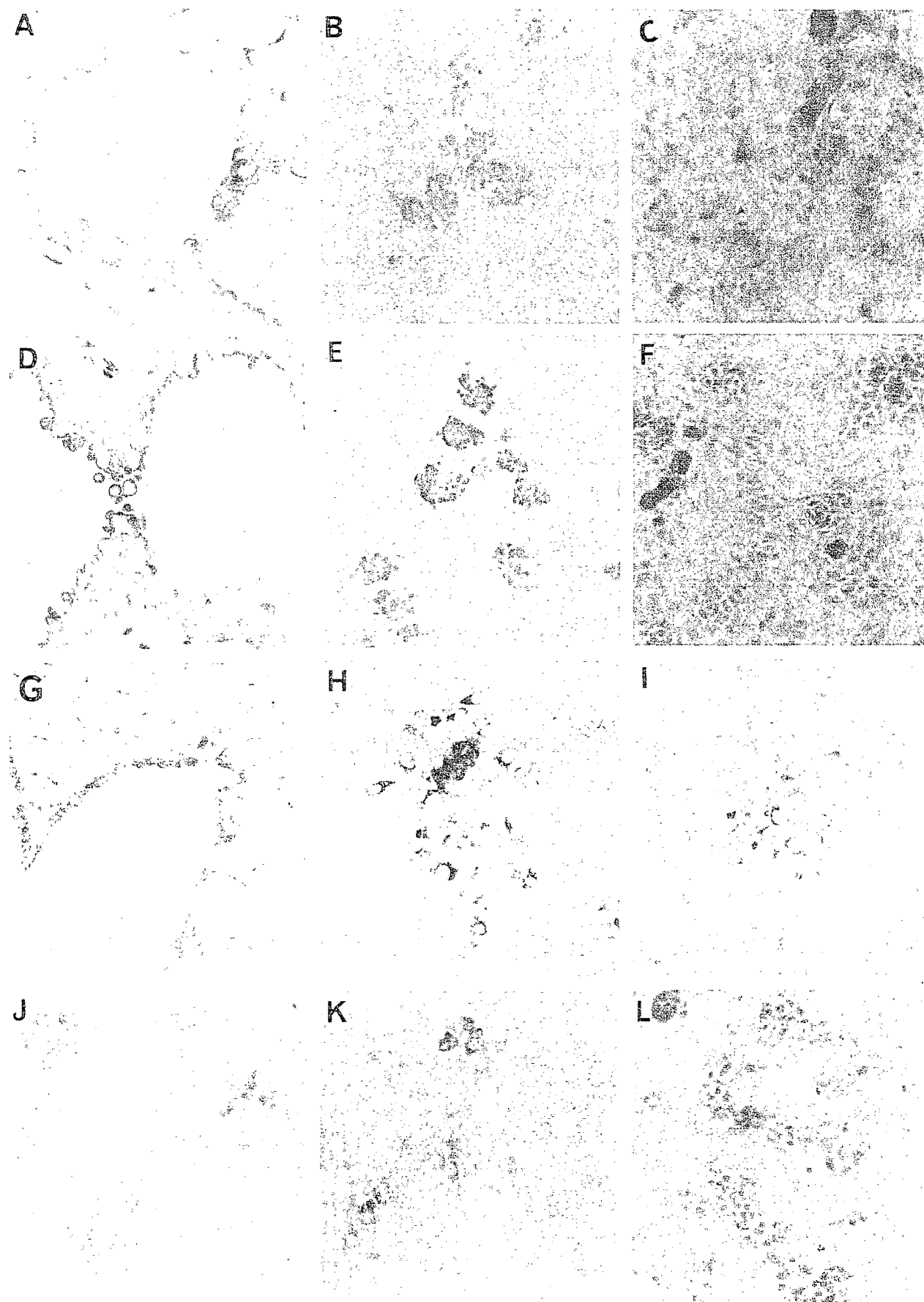
Figure 1. Characterisation of cultured mouse Clara cells. (A) BPA reacted with apical membranes of bronchiolar Clara cells in mouse lung tissue. (B) No BPA reactivity was apparent in freshly isolated (day 0) cells but (C) reactivity was detectable again at day 5 in culture. (D) MPA also reacted with apical membranes of bronchiolar Clara cells in mouse lung tissue. Reactivity was also evident in (E) freshly isolated (day 0) cells and (F) cultured

Table 1. Lectin binding to mouse Clara cells in lung tissue and during culture.

	BPA	ConA	HPA	MPA	WGA	CC10	NBT	Keratin	EM
Lung tissue	++	++++	0	+++	+++	++++	n.d.	n.d.	n.d.
Cells day 0	0	++++	0	++	+++	+++	75%	95%	78%
Cells day 1	+	++++	0	+++	+++	+++	50%	100%	80%
Cells day 5	++	++++	0	+++	+++	++	40%	100%	85%

Note: ++++ = very strongly positive; +++ = strongly positive; ++ = moderately positive; + = weak/occasional positive; 0 = no stain; NBT = nitrotetrazolium blue; EM = electron microscopy; n.d. = not determined; $n=3$.

present within clumps of cells which had not spread fully, rather than in the areas of flattened, spread cells.

Keratin immunocytochemistry

Approximately 95% freshly isolated cells contained keratin, as detected immunocytochemically, indicating their epithelial origin. At day 1 and day 5 (Figure 1L), all cells were keratin positive suggesting that contaminating non-epithelial cells such as macrophages or fibroblasts present in initial isolates had either failed to attach or survive in culture.

Rat type II cells

Lectin binding – lung tissue

BPA reacted strongly with the surface membranes of type I cells in rat alveolar tissue but there was no reaction in the apical membranes of type II cells (Figure 2A). Very strong reactivity was demonstrated with MPA in the membranes of type II cells whereas type I cells did not show reactivity with MPA (Figure 2C). Lectin binding was completely blocked or significantly weakened by control hapten treatment.

Lectin binding – cultured cells

Lectin binding capacity was studied for 8 days and the results are summarised in Table 2. BPA reacted with freshly isolated type II cells and reactivity remained for the duration of the cultivation. MPA reactivity on day 1 was identical with BPA reactivity (Figure 2B and D). MPA membrane staining could be detected on 90–95% of cells, however, intensity significantly declined by day 4. ConA, HPA and WGA bindings varied during the culture period.

Electron microscopy

The purity of the type II cell cultures was similar to that of the Clara cell cultures up to day 4, with over 70% of cells identifiable by EM as type II, that is, containing at least 4 lamellar bodies. However, by day 6, a dramatic reduction in the number of identifiable type II cells occurred, and by day 8, no cells contained lamellar bodies.

Alkaline phosphatase

There was a progressive loss of alkaline phosphatase activity during the culture period with no detectable activity remaining by day 8 (Table 2).

Discussion

Our understanding of the various differentiation pathways operating in lung epithelium is limited. This is due in part to the apparent ability of lung epithelial cells to differentiate along multiple pathways (Johnson *et al.* 1990) and also the occurrence of various 'intermediate' phenotypes, both *in vivo* and *in vitro*, which have features characteristic of more than one cell type, hindering accurate identification (Jeffery & Li 1997). Demonstration of cell surface glycoproteins using lectin histochemistry has been proposed as a possible method for identifying specific lung cells *in vivo* and *in vitro*, and tracking subsequent routes of differentiation which occur, for example, during normal homeostasis, following injury or during disease, or in culture. However, many of the lectin histochemical studies reported in lung epithelial cells to date are conflicting or have not included alternative methods of cell identification for comparison with lectin receptor expression. This makes interpretation of observations difficult. In this study we have attempted to clarify the lectin binding properties of 2 subpopulations of cultured lung cells and to evaluate the potential of lectins as markers of differentiation in these cells. Mouse Clara cells and rat type II cells were cultured for 5 and 8 days respectively. During this time, their capacity to bind each of a panel of 5 lectins was determined and compared with binding to corresponding cells in mouse and rat lung tissue *in situ*. In addition, parallel biological and EM analyses were carried out to provide a comprehensive evaluation of the relevance of lectin receptor expression to cell differentiation in culture.

At the ultrastuctural level, EM analysis indicated that the mouse Clara cell cultures consisted of approximately 80% Clara cells at each time point, as identified by the presence of high numbers of mitochondria, secretory granules and

cells at day 5. CC10 immunoreactivity was present in bronchiolar Clara cells in (G) mouse lung tissue and (H) cultured cells at day 1. However, (I) immunoreactivity had decreased by day 5 of culture. (J) Approximately 75% freshly isolated Clara cells were NBT positive but (K) positivity had decreased by day 5. (L) All cells present in day 5 cultures were keratin positive. Magnification, A and D: ×400; all others, ×200.
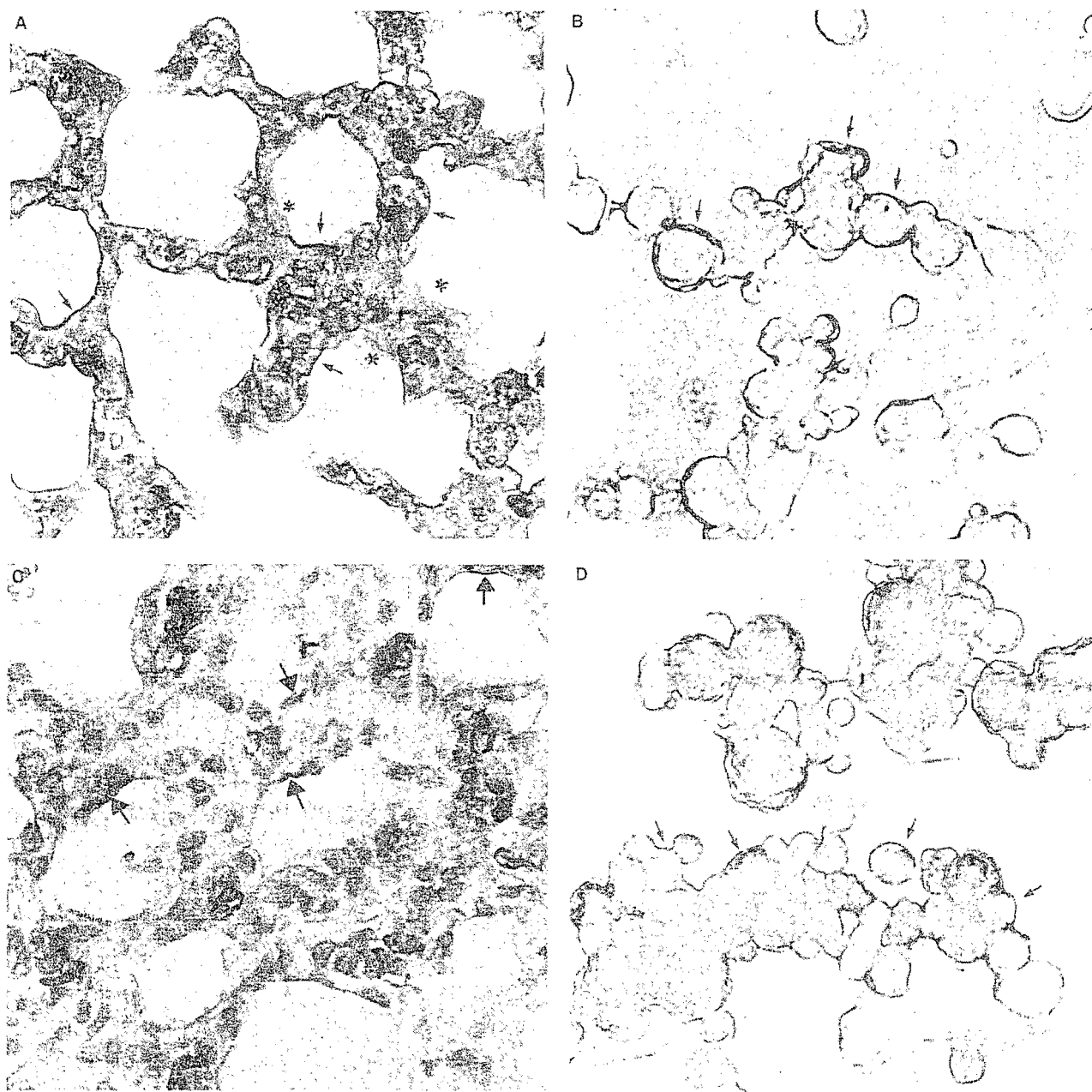
Figure 2. Characterisation of cultured rat type II cells. (A) BPA reacted strongly with apical membranes of alveolar type I cells (arrows) but not type II cells (asterisks) in rat lung tissue. In contrast, (B) BPA reacted with the majority of cultured type II cells (arrows) at day 1. (C) MPA reacted strongly with apical membranes of alveolar type II cells (arrows) in rat lung tissue and (D) reactivity remained at day 1 in culture (arrows). Magnification. A, B and D: ×650; C: ×320.

abundant endoplasmic reticulum. Ciliated cells accounted for almost all of the remaining 20% of cells at each time point. Interestingly, in these EM studies, there was no evidence of Clara cell differentiation over the time in culture. Keratin immunohistochemistry confirmed the lack of contaminating non-epithelial cells in the cultures. However, the decline of NADPH-dependent reductase activity demonstrated by NBT histochemistry and the reduction in CC10 immunoreactivity suggested that the Clara cells were losing at least some of their more specialised functions, a common occurrence in cultured

cells. The reactivity of 4 of the 5 lectins (ConA, MPA, WGA all strongly reactive and HPA non- reactive) was identical when examined in Clara cells *in situ* in mouse lung tissue and in Clara cells cultured for 5 days. Only BPA showed altered reactivity *in vitro* compared to *in vivo*. The loss of BPA reactivity at day 0 and its reappearance at day 1 and day 5 in cultured Clara cells suggests that the BPA-reactive glycoprotein was altered or damaged during the isolation procedure, possibly being cleaved during trypsinisation, but was subsequently repaired or resynthesised in culture.

Table 2. Lectin binding to rat type II cells in lung tissue and during culture.

	BPA	ConA	HPA	MPA	WGA	AP	EM
Lung tissue	0	++++	++	++++	+++	n.d.	n.d.
Cells day 0	+++	++++	++	++++	+	85%	80%
Cells day 1	+++	++++	++	++++	+++	81%	85%
Cells day 2	+++	++++	++	++++	++	50%	71%
Cells day 4	+++	+++	++	++	++	42%	70%
Cells day 6	+++	+++	++++	++	+	5%	8%
Cells day 8	+++	+++	+++	++	+++	0%	0%

Note: ++++ = very strongly positive; +++ = strongly positive; ++ = moderately positive; + = weak/occasional positive; 0 = no stain; AP = alkaline phosphatase activity; EM = electron microscopy; n.d. = not determined; $n = 3$.

These findings indicate that at the ultrastuctural and cell surface glycoprotein levels, Clara cells cultured under the conditions described here closely resemble Clara cells *in vivo*. The repertoire of cell surface glycoproteins of cultured Clara cells was very similar to that *in vivo*. Except for the temporary alteration in BPA reactivity, the composition of the culture medium or the culturing process itself appeared to have no effect on the expression of the glycoproteins involved, or alter their chemical composition to render them unrecognisable by the lectins. Despite this, however, specialised functions such as NADPH-dependent reductase activity and CC10 expression declined in culture. While future optimisation of the culture conditions may improve retention of such functions, it appears that ultrastructural or lectin binding studies alone do not provide an accurate reflection of Clara cell function *in vitro*. Biochemical measurements such as NBT reductase activity, CC10 production and cytochrome p450 activity, for example, should be carried out in parallel.

Cultured rat type II cells, in contrast to mouse Clara cells, reacted with HPA although the reactivity was found to vary during the time in culture. As is already known, ConA binding was not specific to type II cells, but also bound to type I cells. The cells showed variable reactivity with WGA also. Cultured type II cells showed altered reactivity for MPA and BPA compared with type II cells *in vivo*. BPA binding capacity, which we found to be characteristic of type I cells but not type II cells *in vivo*, appeared during the type II cell isolation and its intensity did not change during the culture period. The intensity of MPA binding in the cells decreased by day 4. At the same time, alkaline phosphatase activity, a functional parameter of type II cells, rapidly decreased (only 42% of cells were AP positive at day 4), in contrast to EM analysis which indicated that 70% of cells had the characteristic features of type II cells containing 4 or more lamellar bodies. At day 6, EM findings were more consistent with AP activity (5%) with only 8% of cells containing 4 or more lamellar bodies. By day 8, AP activity had ceased and the cells did not exhibit the characteristic ultrastructure of type II cells.

On the basis of the above results, the appearance of BPA positivity appears to be an early sign of altered surface properties. The decrease in AP activity and MPA reactivity are similarly early and sensitive signs of morphological and functional alterations in type II cells, although at this early stage the cell ultrastructure still resembles type II cells. The aetiology of changes in ConA, HPA and WGA bindings was not dealt with in this study.

The stability of BPA binding and the decrease in MPA reactivity and AP activity suggest that in the course of cultivation, intermediate type cells develop. These intermediate cells differ from *in vivo* type II cells, showing some surface properties (MPA positivity) but revealing also stable oligosaccharide sequences characteristic of type I cells.

It is, therefore, proposed that lectin histochemistry combined with enzyme histochemistry may offer a very sensitive and useful tool for monitoring type II cells in culture as well as *in vivo*.

Acknowledgements

References cited

Brandt AE (1982) Cell surface saccharides of rat lung alveolar type 1 and type 2 cells. *Fed Proc* 41: 755.

Dixon MT, Jersild RA (1983) Changes in lectin binding to the surface of rat lung alveolar epithelial cells. *Am J Pathol* 113: 389–395.

Dobbs LG, Williams MC, Brandt AE (1985) Changes in biochemical characteristics and pattern of lectin binding of alveolar type II cells with time in culture. *Biochim et Biophys Acta* 846: 155–166.

Geleff S, Bock P, Stockinger L (1986) Lectin-binding affinities of the epithelium in the respiratory tract. A light microscopical study of ciliated epithelium in rat, guinea pig, and hamster. *Acta histochem* 78: 83–95.

Hoet PMH, Lewis CPL, Demedts M, Nemery B (1994) Putrescine and paraquat uptake in human lung slices and isolated type II pneumonocytes. *Biochem Pharmacol* 48: 517–524.

Jeffery PK, Li D (1997) Airway mucosa: Secretory cells, mucus and mucin genes. *Eur Respir J* 10: 1655–1662.

Johnson NF, Wilson JS, Habbersett R, Thomasson DG, Shopp GM, Smith DM (1990) Separation and characterisation of basal secretory cells from the rat trachea by flow cytometry. *Cytometry* 11: 395–405.

Joyce-Brady MF, Brody JS (1990) Ontogeny of pulmonary alveolar epithelial markers of differentiation. *Dev Biol* 137: 331–348.