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GENERATION OF AN IMMORTAL DIFFERENTIATED LUNG TYPE-II EPITHELIAL CELL LINE FROM THE ADULT H-2K^{b}tsA58 TRANSGENIC MOUSE

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SUMMARY

This paper describes a new fully differentiated Type-II alveolar epithelial cell line designated T_7, derived from transgenic H-2K^b-tsA58 mice, capable of being passaged as an immortalized cloned cell line in culture. H-2K^b-tsA58 mice harbor a temperature-sensitive (ts) mutant of the simian virus 40 (SV40) large tumor antigen (T antigen) under the control of the γ-interferon (INF)–inducible mouse major histocompatibility complex H-2K^b promoter. When cultured under permissive conditions (33° C and in the presence of γ-INF) cells isolated from H-2K^b-tsA58 mice express the large T antigen, which drives the cells to proliferate. However, upon withdrawal of the γ-INF and transfer of the cells to a higher temperature (39° C), T antigen expression is turned off, the cells stop proliferating and differentiate. The T_7 cell line is a clonal cell line originally derived from a Type-II cell-rich fraction isolated from lungs of H-2K^b-tsA58 mice. The T_7 cells form confluent monolayers, and have a polarized epithelial cell morphology with tight junctions and apical microvilli. In addition, the T_7 cells have distinct cytoplasmic lamellar bodies, which become more numerous and pronounced when the cells are grown under nonpermissive conditions. The T_7 cells synthesize and secrete phosphatidylcholine and the three surfactant proteins, SP-A, SP-B, and SP-C. The T_7 cell line is unique in that it is the first non–tumor-derived Type-II cell line capable of synthesizing and secreting the major components of surfactant. Based on the criteria studied, the T_7 cell line is phenotypically very similar to normal Type-II cells. The T_7 cell line, therefore, should prove a valuable experimental system to advance the study of the cell biology/physiology of surfactant metabolism and secretion as well as serve as a model for other studies of Type-II cell physiology.

Key words: pulmonary; surfactant; lipid; protein; secretion; synthesis.

INTRODUCTION

Lung Type-II epithelial cells are present within the alveolar lining (Coalson, 1994) and produce the surface tension lowering lipoprotein complex, surfactant (Clements, 1957). Surfactant is a complex mixture of phospholipids and three proteins called surfactant protein A (SP-A), B (SP-B), and C (SP-C) (Weaver and Whitsett, 1991). The surfactant complex reduces surface tension at the air–liquid interface in the alveolus, which prevents alveolar collapse during expiration. When the surfactant is absent or is deficient, as in premature infants or in certain pathological states in adults, respiratory distress ensues (Avery and Mead, 1959; Hartog et al., 1995).

The study of the cell biology and biochemistry of surfactant synthesis and secretion has been severely hampered due to the lack of a suitable in vitro cell model. One can isolate and culture primary Type-II cells; however, this eventually results in the loss of differentiated characteristics, including production of phospholipids and surfactant proteins (Dobbs, 1990; Leslie et al., 1993). Several Type-II cell lines have been derived from animals and human tissue (Smith, 1977; O'Reilly et al., 1988a; Mallampalli et al., 1992; Steele et al., 1992; Wikenheiser et al., 1993; Sen et al., 1994), but generally these have originated from tumors and most do not manifest sustained secretion of lamellar bodies, phospholipids, or the surfactant proteins. None of these cell lines are phenotypically close to Type-II cells. Moreover because of their transformed origin, it is uncertain whether these cell lines accurately reflect the cell biology/physiology of normal Type-II cells.

Polarized epithelial cells are notoriously hard to culture. However, using the transgenic H-2K^b-tsA58 (Immorto) mouse (Jat et al., 1991), several groups have recently generated differentiated immortal cell lines from epithelial tissues, such as colonic and small bowel epithelium (Whitehead et al., 1993), and bone (osteoclasts) (Chambers et al., 1993). These cell types had hitherto proved difficult, if not impossible, to culture. The transgenic H-2K-tsA58 (Immorto) mouse has inserted into its genome a construct consisting of the 5′ flanking promoter sequence and the transcriptional initiation site of the mouse major histocompatibility complex H-2K^b class I (MHC) gene coupled to the coding sequence of a temperature-sensitive form of the simian virus 40 (SV40) T antigen (Jat et al., 1991). The MHC gene promoter is not only widely active in a range of tissues but is also susceptible to additional induction by interferons. When cells from the Immorto mouse are isolated and grown under permissive conditions (33° C and with γ-interferon [γ-INF] present in the media) the SV40 T antigen is expressed causing the cells to

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proliferate. However, upon withdrawal of the γ-INF and transfer of the cells to a higher temperature (39° C), T antigen expression is turned off, the cells stop dividing, and, they proceed to differentiate.

In this paper we describe the generation and characterization of the T_7 cell line, the first non–tumor-derived immmortalized, fully differentiated Type-II cell line. This cell line was produced by dilution cloning of a Type-II cell-rich fraction produced from the lungs of transgenic H-2K^b-tsA58 (Immorto) mice. The T_7 cell line is novel in that it expresses SP-A, SP-B, and SP-C. Furthermore, the T_7 cells secrete all three of these proteins plus phosphatidylcholine, the major lipid component of the surfactant. In addition, the cells have a polarized epithelial cell morphology with distinct cytoplasmic lamellar bodies. The T_7 cell line is phenotypically close to normal Type-II cells; therefore they will prove a valuable tool for the study of Type-II cell physiology.

Materials and Methods

Isolation and immortalization of Type-II epithelial cells from the lungs of transgenic H-2K^b-tsA58 mice. A modification of the technique described by Mason et al. (1997b) was used to isolate Type-II cells from the Immorto transgenic mouse lungs (Jat et al., 1991). In preliminary experiments on the parent nontransgenic strain CBA/Ca × C57BL, it was determined that a minimum of four mouse lungs were necessary to obtain a visible cell pellet. Consequently four Immorto mice were anesthetized and heparinized. The trachea was cannulated and the aorta transected to exsanguinate the mice while ventilation was maintained. The lungs were perfused through the pulmonary artery with a dextrose/salt solution until they appeared white and blood-free. The dextrose/salt solution was then used to lavage the lungs seven times, followed by lavage twice with a dextrose/salt solution plus 1.9 m*M* calcium chloride ($CaCl_2$) and 1.2 m*M* magnesium sulfate ($MgSO_4$). A 20-min incubation at 37° C followed, while the trachea was filled with the latter solution plus elastase. The airways and nonparenchymal tissues were dissected away and the rest of the lung placed in a deoxyribonuclease solution and minced into 1-mm cubes. Elastase activity was stopped with the addition of fetal bovine serum. The cell suspension was filtered through Teflon meshes of 100 and 20 μm, underlaid with fetal bovine serum and centrifuged at 130 × *g* for 8 min. The lighter Type-II cells remain in the supernate. The supernate was again centrifuged to obtain a cell-rich pellet. To remove contaminating macrophages this pellet was resuspended in serum-free medium, placed on IgG-coated dishes and incubated for 1 h at 37° C. The supernate from the IgG-coated dishes was then centrifuged at 130 × *g* for 8 min. The Type-II cell-rich pellets were resuspended in Dulbecco modified Eagle medium (DMEM) containing: fetal bovine serum, 2%; penicillin, 100 U/ml; streptomycin, 1%; amphotericin B, 250 μg/ml; γ-INF, 100 U/ml; transferrin, 5 μg/ml; epidermal growth factor, 0.025 μg/ml; insulin, 10 μg/ml; endothelial cell growth supplement, 7.5 μg/ml; endothelin-1, 40 nmol/ml; T3, 20 ng/ml; hydrocortisone, 0.36 μg/ml. These additives were selected because they are known to promote Type-II cell differentiation (Kresch et al., 1987; Massey et al., 1987; Van Scott et al., 1988; Mason et al., 1990; Sen et al., 1994). The cells were then plated on tissue culture plastic in low volume to promote cell adherence. Cells were incubated overnight in 10% CO_2 at 33° C, the permissive temperature, which promotes cell proliferation. The cells grew rapidly to confluence on tissue culture plastic, and after trypsinization were split and replated. Cells were passaged eight times and then clonal cell lines were derived by limiting dilution cloning (Freshney, 1991). Cells grown at the time of cloning were designated as passage 1.

For some experiments, cells were grown on collagen IV-coated plastic or on collagen-coated transwells (Becton Dickinson, Bedford, MA). For these experiments, the medium was supplemented with 2% Ultroser G (Biosepra, Cedex, France) (Yamaya et al., 1992). To study the effects of growth with an apical air surface on cell phenotype, cells were grown on collagen gels prepared as described by Dobbs et al. (1997). Briefly, a stock solution of bovine dermal collagen (Vitrogen, Santa Clara, CA) was prepared by adding to eight parts of bovine collagen, one part of 10× minimal essential medium (MEM, GIBCO, Grand Island, NY), and 1.4 ml of sterile 0.4 *N* NaOH. After placing 3 ml of the collagen stock solution on 30-mm Millicell CM membranes (Millipore, Bedford, MA), the collagen was allowed to polymerize for 2 h at 37° C in a 5% CO_2 incubator. The membranes were washed six times with MEM before plating with cells. After growth for ~48 h, the medium from the top of the collagen gels was removed to expose the apical cell surface to air. Cells were grown for 24–48 h under these conditions before harvesting for electron microscopy.

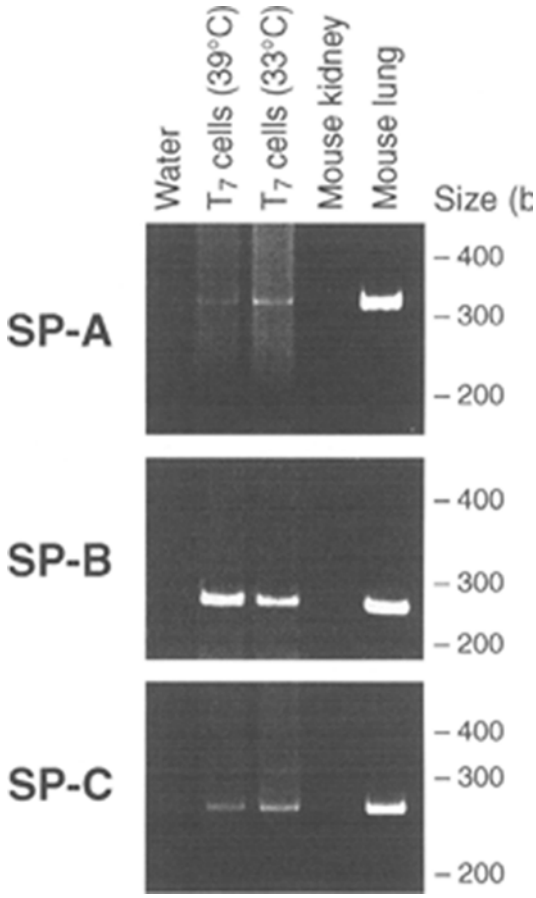

FIG. 1. RT-PCR was done on total RNA extracted by cesium chloride gradient from the T_7 clone. *Lane 1* is a negative control (without RNA) and represents no PCR product of the RT-PCR reaction without RNA. *Lane 2* represents the PCR product resulting from the inclusion of RNA from T_7 cells grown on tissue culture plastic at 33° C in the presence of γ-INF for ~5 d and then transferred to 39° C for 3 d without γ-INF. In *lane 3*, RNA from T_7 cells grown to confluence on tissue culture plastic at 33° C in the presence of γ-INF was used. In *lanes 4* and *5*, RNA from mouse kidney or lung representing negative and positive tissue controls, respectively, was included in the PCR mixture. The amount of RNA used in each lane was standardized by optical density taken at 260 nm. In *panel A*, the expected 346 bp SP-A cDNA fragment was amplified from RNA from the T_7 clone and from mouse lung. No SP-A cDNA amplification is seen in the negative *lanes 1* or *4*. In *panel B* the expected SP-B cDNA 260-bp fragment is seen in the T_7 *lanes 2* and *3* and in *lane 5*, the positive control, but not in the negative control *lanes 1* and *4*. In *panel C* the expected SP-C cDNA 251-bp fragment is seen in the T_7 *lanes 2* and *3* and in the positive control *lane 5*, but not in the negative control *lanes 1* and *4*. In all three panels an expected higher molecular weight DNA band was observed in *lanes 2–4* reflecting contamination of RNA with genomic DNA and in *lane 4* serves to show successful amplification in this negative control. Using primers for mouse β-actin cDNA, an expected 500-bp fragment was detected in cells corresponding to *lanes 2–5* (data not shown).

5-Bromo-2′-deoxy-uridine incorporation. The incorporation of 5-bromo-2′-deoxy-uridine (BrdU) into cellular DNA was detected using an immunocytochemical assay kit from Boehringer Mannheim Biochemicals (Indianapolis, IN). T_7 cells were grown in slide chambers (Nunc, Inc., Naperville, IL) at 33° C with γ-INF (permissive conditions) for 5 d, or under permissive conditions for 48 h and then transferred to nonpermissive conditions (39° C

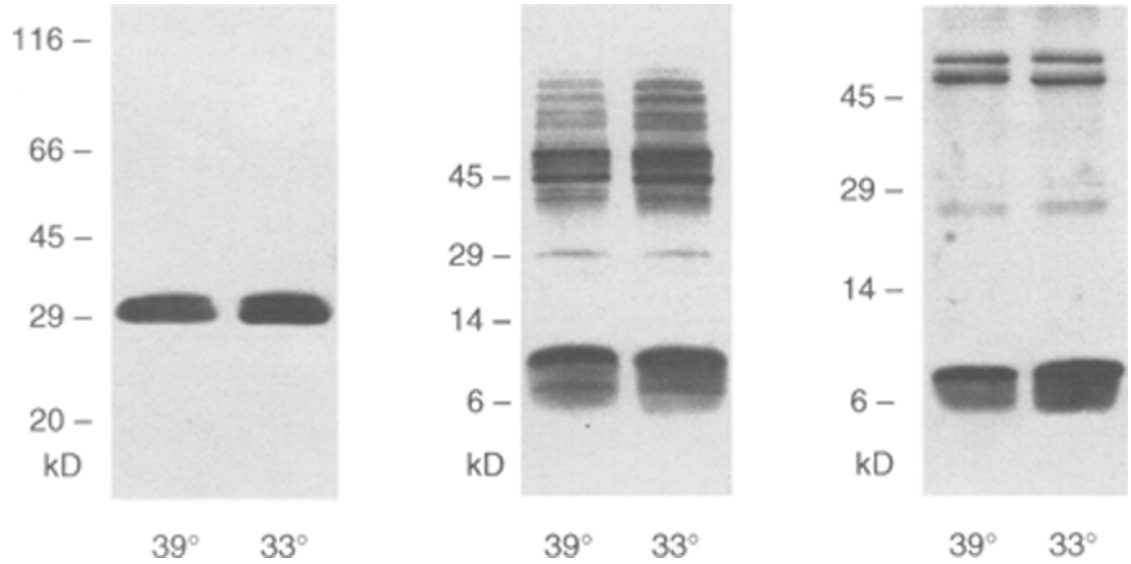

FIG. 2. Western blot analysis of cell lysates from T_7 cells grown on tissue culture plastic at 39 or 33° C for expression of surfactant protein SP-A, SP-B, and SP-C. In *panel A*, cell lysates were electrophoresed in 10% polyacrylamide/SDS-PAGE and the blot probed with a polyclonal antiserum to SP-A (O'Reilly et al., 1991). A 26–30-kD SP-A band was detected. In *panel B*, T_7 cell lysates were electrophoresed in 15% polyacrylamide/SDS-PAGE and the blot probed with a monoclonal antibody to porcine SP-B (Smith, 1977). A 42–44 kD SP-B pro-protein band was seen in addition to a 28-kD-translational product of SP-B, and an 8-kD petide band, which is the size of the mature processed SP-B protein. In *panel C*, the cell lysates were electrophoresed in 15% polyacrylamide/SDS-PAGE and the blot probed with murine pro–SP-C specific antiserum (Beers et al., 1992, 1994). A band of ~46 kD was seen, which represents a technical artifact resulting from multimer formation by the extremely hydrophobic SP-C protein. In addition, a 6–8-kD processing product of SP-C was seen.

without γ-INF for 3 d). Five-day old cultures were then pulsed with 10 μmol/L BrdU for 1 h before staining with anti-BrdU antibody crosslinked with alkaline phosphatase. The cells with BrdU incorporation as evidenced by nuclear staining were counted for both growth conditions.

Transepithelial resistance and impermeability of the T_7 cell monolayer. Electrical measurements to determine transepithelial resistance were performed on cells plated on collagen-coated transwells that had achieved confluence. The Millicell ERS voltmeter (Millipore) was used, and first, the resistance was measured in a 'blank', i.e., across a transwell membrane with no cells but with the same volume of medium in the top and bottom wells. The resistance was then measured across a confluent monolayer of cells grown on a transwell membrane. The difference between the readings in the blank and the cell sample was taken as the transepithelial resistance. In addition, the impermeability of the confluent monolayer was demonstrated using tritiated inulin. After the addition of inulin (650 μCi/ml) to the medium in the top well, the transwells were incubated for 1 h before aliquots of medium present in the apical and basal wells were taken. The amount of inulin present in each aliquot of medium was then determined by liquid scintillation counting.

RNA extraction and reverse transcriptase-polymerase chain reaction. Each clonally isolated, conditionally immortalized, cell line was grown to confluence in two 100-mm dishes and total cellular RNA was isolated according to the method of Chomczynski and Sacchi (1987) and by subsequent cesium chloride gradient centrifugation. RNAs similarly prepared from mouse lung and mouse kidney (Ambion Inc., Austin, TX) were used as positive and negative controls, respectively. The cDNA Cycle Kit (Invitrogen, San Diego, CA) was used for the reverse transcription (RT) of 0.1–1.0 μg of RNA at 42° C for 1 h with either oligo-deoxythymidine or specific antisense oligonucleotide primers, and the RT reaction was stopped with the addition of 1 μl of 0.5 *M* ethylenediamine-tetraacetic acid. Four microliters of the RT mix was removed for each polymerase chain reaction (PCR) assay. The specific sense and antisense oligonucleotides used for RT-PCR of the murine SP-A, SP-B, SP-C, and β-actin genes are given as follows, with each pair chosen to span the indicated intron and to yield, by PCR, an amplified fragment of the indicated size in bp: (SP-A) sense 5′ TACCTGGATGAGGAGCTTCA 3′ intron 3, antisense 5′ TCCTGGGTACCAGTTGGTGT 3′, 346 bp; (SP-B) sense 5′ AGGACTTCTCTGAGCAACAG 3′, intron 6, antisense 5′ ATGGCATCCTCAGTGGAAGA 3′, 260 bp; (SP-C) sense 5′ CTCCTGACGGCCTATAAGCC 3′, intron 4, antisense 5′ TAGTAGAGTGGTAGCTCTCC 3′, 251 bp; (β-actin) sense 5′ CGTACCACGGGCATTGTGAT 3′, antisense 5′ GAGCAGTAATCTCCTTCTGC 3′, 500 bp. PCR was performed for 40 cycles with denaturing for 1 min at 94° C, annealing for 2 min at 58° C (55° C for SP-B), and extension for 2 min at 72° C. The PCR products were analyzed by 10% polyacrylamide gel electrophoresis and ethidium bromide staining. A clone, T_7, which expressed all four mRNAs including SP-C, as determined by RT-PCR, was chosen for further characterization.

Synthesis of SP-A, SP-B, and SP-C by T_7 Type-II cell clone demonstrated by immunoblotting. T_7 cells were grown on collagen IV-coated transwell filters under permissive conditions to near confluence, when some were transferred to nonpermissive conditions for 48–72 h. For some experiments, cells were treated with different doses of γ-INF—25, 50, or 100 μg/ml—when placed in permissive conditions, and after growth for 48–72 h or until near confluence, transferred to nonpermissive conditions. Cells were then harvested for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). For positive controls whole mouse lung homogenates were used. For each sample the protein concentration was determined by spectrophotometric comparison with known standards (Lowry et al., 1951). Thus, an equal amount of protein from each sample was loaded and electrophoresed on one-dimensional 10 or 15% SDS-PAGE gels. Gels were electrophoretically transferred to 0.2-μm nitrocellulose (Hybond ECL, Amersham, Buckinghamshire, England), at 150 mA/cm² for 1.5 h, using a mini transblot electrophoretic transfer cell (BioRad, Hercules, CA). After overnight blocking at 4° C, filters were incubated at room temperature for 2 h in primary antisera at titers determined as optimal by preliminary experiments for each antiserum used. The antiserum for SP-A was kindly provided by Dr. David Phelps (Phelps et al., 1984). This affinity-purified antibody raised in rabbits against human alveolar proteinosis extract recognizes the 35 kDa SP-A and crossreacts with SP-A derived from the rat. The antibody to SP-B was a generous gift from Dr. Yasuhiro Suzuki (Suzuki et al., 1986) and was prepared in a hybrid cell (8B5E) prepared by

fusing a myeloma cell X63.Ag8.653 with spleen cells of the Balb/c mouse immunized with a surface-active fraction of porcine lung lavage. The antibody recognizes the 15 kDa SP-B and crossreacts with the murine-derived antigen. A second antibody to purified SP-B was obtained from the laboratory of Dr. Jeffrey Whitsett (Lin et al., 1996). This antiserum raised in rabbits recognizes the mature 8 kDa SP-B peptide. The antiserum to SP-C was kindly provided by Dr. Michael F. Beers (Beers et al., 1992, 1994). It was raised in rabbits against purified synthetic NproSP-C peptide and recognizes the 20–21 kDa, 16 and 6 kDa translational products of SP-C.

The incubation with the primary antiserum was followed by incubation with secondary goat anti-rabbit or anti-mouse antisera (depending on the primary antibody), conjugated to horseradish peroxidase (Amersham Corp., Arlington Heights, IL) at titers ranging from 1:5000 to 1:24,000 for 1 h. Bands were visualized by enhanced chemiluminescence (ECL) using the ECL kit (Amersham). To test for nonspecific reactivity of the secondary antibody with mouse antigens, some immunoblots were incubated with each secondary antibody, alone, for the same duration and at the same titer as used for the immunoblotting after the primary incubation. These immunoblots were then reacted with the ECL kit reagents to visualize any bands that resulted from nonspecific staining; no such nonspecific bands were seen.

Secretion of surfactant proteins SP-A, SP-B, and SP-C by T_7 cell clone. Cells were grown on collagen IV-coated 6-well tissue culture plates, as described above, for 48–72 h. After transfer to nonpermissive conditions, the medium was collected and centrifuged at 200 × *g* for 10 min to pellet any floating cells. The supernatant was then transferred to a fresh tube and concentrated to one-third the volume in a speed vacuum concentrator. The concentrate was then boiled with SDS-PAGE loading buffer and separated on a 10% SDS-PAGE gel as described above. The separated proteins were then electroblotted onto a nitrocellulose membrane. The blot was then cut into strips; each strip was then probed with an antibody raised against SP-A, SP-B, or SP-C.

Electron microscopy. For electron microscopy, cells were grown to confluence in a 100 mm Petri-dish, on collagen-coated plastic, in transwells (Becton Dickinson) or on collagen gels as described above. The cells were grown for 48 h at 33° C in medium supplemented with γ-INF, and then for 48 h at 39° C in medium without γ-INF. The growth medium was aspirated and the cells rinsed with phosphate-buffered saline. This was discarded and the cells were overlaid with 2% glutaraldehyde for 2 h. In some instances, the growth medium was changed 4–6 h before the cells were harvested. The cells were then osmicated, gently scraped with a rubber policeman, and processed in acetone followed by embedding in epon. In the case of transwells and collagen gels, the membranes or gels were cut out of the wells, diced, processed, and embedded on end in spurr or epon. Thin sections of cells were stained with uranyl acetate and lead citrate and examined in a JEOL 100S electron microscope. Photographs (8 × 10 in.) of cells at a magnification of ×19,800 were used to compare the number of lamellar bodies in cells grown under permissive or nonpermissive conditions. Photographs of cells were taken at random, and only complete cells were used for the lamellar body count. Circular cytoplasmic structures with multiple parallel membranous lamellae were counted as lamellar bodies.

Secretion of phosphatidylcholine by the T_7 cell clone. T_7 cells were plated onto collagen IV-coated 6-well tissue culture plates, and grown under permissive conditions for approximately 48 h. The medium was then replaced and the cells grown under nonpermissive conditions. After 48 h, 1 μCi/ml [^{3}H]-choline was added to the medium and the cells cultured for a further 24 h to label the phosphatidylcholine pool. The cells were then washed three times with fresh DMEM to remove all traces of unincorporated [^{3}H]-choline. Fresh DMEM (1.5 ml/well) was then added, and the cells returned to the incubator. After 3 h the medium was removed and centrifuged at 200 × *g* for 10 min to remove any floating cells. Lipids were then extracted from the supernatant and cells using the method of Bligh and Dyer (1959), and the total lipid radioactivity determined by liquid scintillation counting. Secretion is expressed as the amount of lipid radioactivity in the medium after 3 h as a percentage of the total in the cells and medium combined. As a control we did determine, by thin layer chromatography (Touchstone et al., 1979), the biochemical nature of the lipids extracted from the medium. As expected the only phospholipid detected in the medium was phosphatidylcholine, and 94–98% of the radioactive label detected in the medium samples was present in the form of phosphatidylcholine.

Results

Identification of a Type-II cell line with expression of surfactant proteins SP-A, SP-B, and SP-C. A Type-II cell-rich fraction was isolated from lungs of adult transgenic H-2K^b-tsA58 mice and placed in primary culture. The cells were passaged eight times before clonal cell lines were derived by limiting dilution cloning. To determine which of the clonal cell lines possessed a Type-II cell-like phenotype, candidate clones were screened by RT-PCR for the expression of messenger RNA for SP-A, SP-B, and SP-C. Primer pairs were selected from the published cDNA and genomic sequences of SP-A (Korfhagen et al., 1992), SP-B (D'Amore-Bruno et al., 1991), and SP-C (Glasser et al., 1990). Each pair of primers spans a specific intron so that any genomic DNA contaminating the RNA preparation would produce a larger amplified DNA fragment than the expected cDNA fragment using the same primers. RT-PCR was also performed with primers from the mouse β-actin gene (Tokunaga et al., 1986) in order to verify the presence and integrity of the RNA samples. A number of the candidate Type-II clones were shown to express each of the three surfactant mRNAs (data not shown). One clone, designated T_7, expressed the highest levels of mRNA for all three surfactant proteins and was chosen for further characterization. The results of the RT-PCR assays on this clone are shown in Fig. 1.

Synthesis of surfactant proteins SP-A, SP-B, and SP-C by T_7 cell clone. Having established that the T_7 clone expressed mRNA for all three surfactant proteins, we next determined whether these mRNAs were translated. To accomplish this, we screened, by Western blot analysis, T_7 cell lysates for SP-A, SP-B, and SP-C proteins (Fig. 2). For these studies, mouse lung homogenates and a lamellar body preparation were used as positive controls. In addition, the antibodies used were fully characterized (*see* Methods section) and are monospecific for their designated antigen. The T_7 cell lysates yielded a single 26–30 kDa band with the SP-A antiserum (Phelps et al., 1984). This band represents the mature fully processed form of SP-A. A band of similar size was observed as the mature SP-A protein in both the mouse lung and lamellar body preparations (data not shown). Both lung and lamellar body preparations revealed several larger molecular weight forms of SP-A as previously published (Phelps et al., 1984). In comparison, with antiserum to SP-B (Suzuki et al., 1986), the T_7 cell lysate yielded a doublet at 42–44 kDa and a diffuse single band at 8–10 kDa. The doublet is the unprocessed pro-form of SP-B, while the 8–10 kDa band represents the mature fully processed SP-B peptide. In the SP-B blots a minor band was also observed at 28–29 kDa, this possibly is the carboxy-terminus of the pro-protein liberated by the first proteolytic cleavage of the unprocessed SP-B protein. All four of these SP-B protein bands were observed with this primary antibody in the mouse lung preparation (data not shown). Finally, with the SP-C antiserum, the T_7 cell lysates yielded a doublet at 46–50 kDa and a very prominent band at 6–8 kDa. Both the mouse lung homogenate and lamellar body positive controls yielded a similar pattern of SP-C proteins (data not shown) as previously described (Beers et al., 1994). The 46–50 kDa band represents a multimer resistant to SDS dissociation that develops in vitro after boiling because of the extreme hydrophobicity of SP-C. A similar band was also detected by Beers et al (1994) and immunoreactivity for this product was eliminated by competition with purified SP-C (Beers et al., 1994). The 6–8 kDa band represents a partially processed form of SP-C. Increased pro-

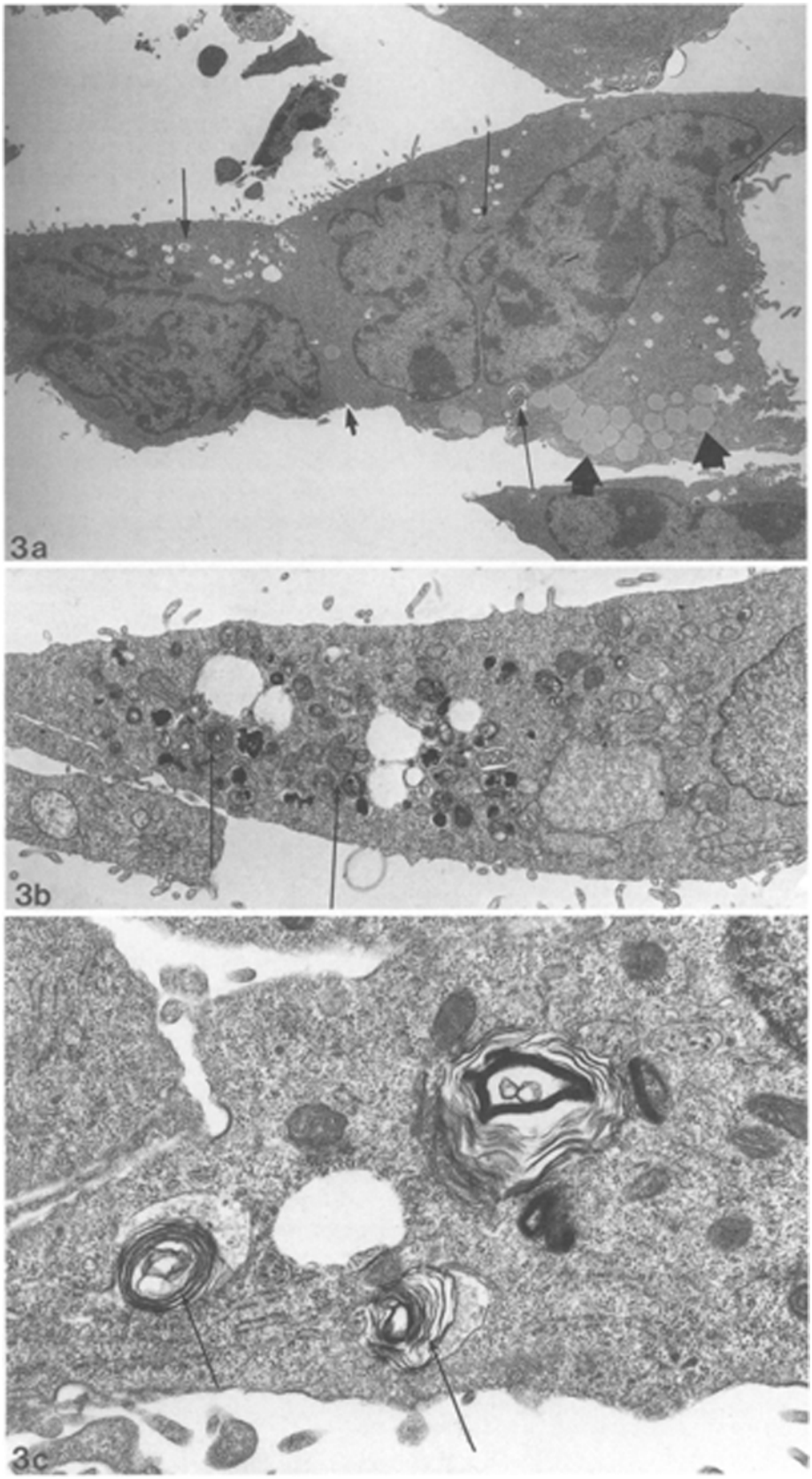

FIG. 3. Transmission electron micrographs of T_7 cells grown on tissue culture plastic. (*a*) Cell growth under permissive conditions at 33° C for 72 h; reveals morphologic polarization characterized by apical filopodia and a smooth basal surface. The cells form intercellular junctions (*short arrow*) and contain cytoplasmic lamellar bodies (*thin arrows*), and basal lipid droplets (*thick arrows*, original magnification

tein expression was seen in cells grown under nonpermissive conditions when a dose of 50 μg/ml of γ-INF was used to stimulate proliferation, and remained unchanged in cells grown under permissive or nonpermissive conditions and over several cell passages (5th through 85th, the latter being the last passage studied).

BrdU incorporation. T_7 cells at both permissive and nonpermissive growth conditions were visualized by alkaline phosphatase–linked antibody to BrdU following BrdU incubation, in order to determine their level of DNA synthesis as a corollary to cell division. Cell proliferation was reduced by nearly 50% as evidenced by reduced BrdU incorporation in cells grown under nonpermissive conditions (mean 56 ± 8%; standard deviation , N = 6), at 33° C; mean 35 ± 6% (SD, N = 5) at 39° C.

Transepithelial resistance and impermeability of the T_7 cell monolayer. The integrity of the T_7 cell monolayer was assessed both by transmembrane electrical resistance and by impermeability to the inert radioactive macromolecule inulin.

The transepithelial resistance measurements ranged from 258 Ω cm^2 at 33° C to 145 Ω cm^2 at 39° C, depending on the degree of cell confluence. There was no leakage of radioactive inulin from the top to the bottom well even after 1 h incubation of medium in the top well with radioactive inulin. Both these findings indicate that the T_7 cells form a confluent monolayer with intact tight junctions.

T_7 cell morphology. Next, we examined the morphology of the T_7 cells by light microscopy. The T_7 cells maintained a polygonal epithelial cell morphology. The nuclei were round to oval, and the cytoplasm was granular. The granularity became noticeably more pronounced when the cells were grown under nonpermissive conditions (data not shown). When examined by electron microscopy the cells appeared to be polarized with apical microvilli and a smooth basolateral surface (Fig. 3). The T_7 cells formed tight junctions and contained cytoplasmic multivesicular and lamellar bodies. The number of lamellar bodies increased approximately fourfold when the cells were switched from permissive to nonpermissive culture conditions (mean at 33° C = 0.82 ± 1.28 and SD, N = 29 lamellar bodies/cell; mean at 39° C = 3.6 ± 2.5 and SD, N = 29 lamellar bodies/cell). In addition, lamellar bodies were significantly more numerous in cells that were exposed to fresh medium 4–6 h prior to harvesting.

Secretion of surfactant proteins SP-A, SP-B, and SP-C by T_7 cell clone. To determine whether the T_7 cells are capable of secreting the three surfactant proteins, they were grown under permissive and nonpermissive conditions; the cells were then washed and incubated in fresh media for 24–48 h. The media was then removed, concentrated, and screened, by Western blot analysis, for SP-A, SP-B, and SP-C (Fig. 4). The T_7 cells secreted all three surfactant proteins. The secreted form of SP-A appeared to have a higher molecular weight than that present in the lysates. This increase in size most likely reflects asparagine-linked glycosylation of the mature polypeptide (Weaver and Whitsett, 1991). In addition to mature fully processed SP-B, the cells also secreted approximately five other immunologically related proteins, ranging in molecular weight from 45–16 kDa, that were recognized by the SP-B antibody. These additional bands probably represent the processing intermediates. For example, the 45 kDa protein is probably unprocessed pro–SP-B, while the 30–32 kDa protein is the clipped carboxy-terminus of the pro-protein. The T_7 cells predominantly secrete the 6–8 kDa partially processed form of SP-C. Cell growth under nonpermissive conditions did not alter secretion of SP-A or SP-B, but for SP-C, a minor band, the 3–5 kDa peptide appeared. This small molecular weight band may represent additional processing of SP-C, although the SP-C antibody used does not recognize the mature form of SP-C resulting from cleavage at the aminoterminus (M. Beers, pers. comm.).

Secretion of phosphatidylcholine by the T_7 cell clone. Finally we determined whether the T_7 cells could secrete phosphatidylcholine, the major lipid component of surfactant. T_7 cells labeled with ^{3}H-choline for approximately 24 h under permissive or nonpermissive culture conditions were washed to remove unincorporated radiolabel and incubated for 3 h in fresh medium. Details of the labeling protocol and secretion experiment are given in the Methods section. In 3 h, the T_7 cells cultured under nonpermissive or permissive conditions secreted 1.53 ± 0.35% (SD, N = 5) or 1.32 ± 0.55% (SD, N = 5) of total cellular ^{3}H into the medium, respectively. As a control, we determined the biochemical nature of the secreted labeled material. On analysis, 94–98% of the secreted ^{3}H label was present in the form of phosphatidylcholine.

Discussion

The study of the detailed cell biology/physiology of the lung Type-II epithelial cell has been severely hampered by the lack of a reliable in vitro cell model. Freshly isolated Type-II cells can be placed in primary culture, but within 24–48 h these cells begin to dedifferentiate and lose their ability to manufacture and secrete surfactant (Shannon et al., 1992; Mason et al., 1997a). New primary culture methodologies have been developed; however, even with these, Type-II cells can only be kept differentiated and viable for 2–4 wk. Thereafter, the cells reach senescence and apoptose. Although the new generation of primary culture methodologies offers many advantages over the older systems, primary cultures are not clonal and are not very hardy and often cannot withstand complex experimental manipulation. Many of these problems could be overcome if a suitable immortalized Type-II cell line existed.

Numerous attempts have been made to establish a differentiated respiratory Type-II cell line; however, these have only had a limited amount of success. In the rat, alveolar Type-II and pre–Type-II cell lines have been developed by transfer of the adenovirus E1A oncogene using a retroviral construct (Smith, 1977; Mallampalli et al., 1992). These cells do not manifest a sustained secretion of lamellar bodies, phosphatidylcholine, or the SP's A, B, or C. Several human Type-II cell lines have been developed from pulmonary adenocarcinomas (O'Reilly et al., 1988a). The NCI-H820 cell line reportedly contains lamellar bodies and synthesizes SP-A, SP-B, and SP-C,

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×6000). (*b*) Cell growth under nonpermissive conditions at 39° C for 48 h; produces an increase in cytoplasmic vesicles and lamellar bodies (*thin arrows*) signaling increased metabolic activity (original magnification ×11,550). (*c*) Cells grown at 39° C for 48 h and harvested ~4 h after fresh medium was added to culture dishes; reveals an increased number of cytoplasmic lamellar bodies (original magnification ×35,000).

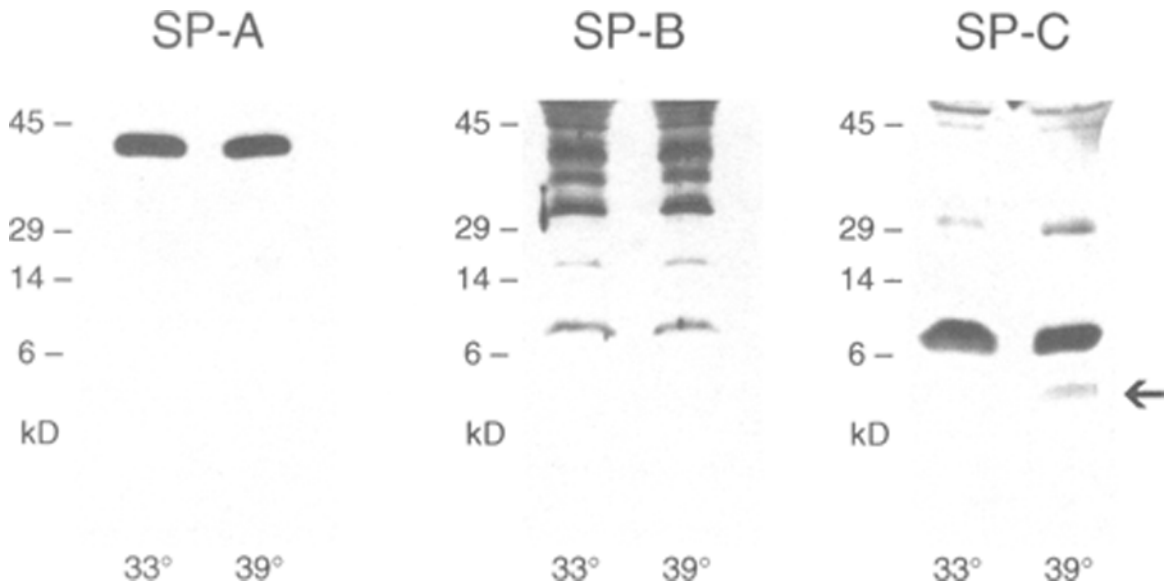

FIG. 4. T_7 cells were grown on collagen IV-coated tissue culture plastic under permissive or nonpermissive conditions for ~96 h. The medium was harvested in the presence of protease inhibitors and spun at 200 × g for 10 min to eliminate any detached cells. The supernatant was transferred to a fresh tube and reduced to one-third the volume in a speed vacuum centrifuge. The concentrated medium was then electrophoresed on 15% polyacrylamide SDS-PAGE and blotted. The blot was cut into three strips and each strip was probed with antibody to SP-A, SP-B, or SP-C as described above. A 26–35-kD band was seen with the antibody to SP-A. Translational products of SP-B including 42–44-kD and 16-kD bands were seen with the antibody to SP-B. In addition, the mature 8 kD SP-B peptide was present, indicating complete processing of SP-B by this cell line. With the antibody to SP-C, the 8-kD peptide was seen in medium from cells grown under both conditions. In addition, cells grown under nonpermissive conditions showed additional processing of SP-C to a 3–5-kD peptide (*arrow*).

but forms nonadherent cultures and so is not appropriate for studies examining secretory polarity. Furthermore, these cells are difficult to grow (Steele et al., 1992). The A549 cell line demonstrates some of the morphologic features of Type-II cells, but does not express surfactant proteins (Smith, 1977). The H441 cell line synthesizes only SP-A and SP-B, but not SP-C, so it is not clear that it really represents a Type-II cell line, as SP-C is considered a marker for Type-II cells (O'Reilly et al., 1988a, 1988b). Recently, distal respiratory epithelial cell lines from tumors generated in transgenic mice harboring the viral oncogene SV40 large tumor antigen under the transcriptional control of a promoter region from the human SP-C gene, have been developed (Wikenheiser et al., 1993). Of the several cell lines reported, the MLE-7 cell line reportedly manifests some morphologic features of Type-II cells and expression of SP-A, SP-B and SP-C. Since these lines were derived from lung tumors, aberrant development is a concern and these cell lines may not accurately reflect the cell biology of normal Type-II cells.

In the study reported here, we have employed the transgenic H-2K^b-tsA58 (Immorto) mouse (Jat et al., 1991) to generate a novel, fully differentiated Type-II cell line. The Immorto mouse harbors within its genome a transgene consisting of the 5′ flanking promoter sequence and the transcriptional initiation site of the mouse major histocompatibility complex H-2K^b class I (MHC) gene fused to the coding sequence of a temperature-sensitive mutant of the SV40 T antigen (SV40tsA58). The MHC gene is active in the majority of cells and is also susceptible to additional induction by interferons. Thus, when cells from the Immorto mouse are isolated and grown under permissive conditions (33° C and with γ-INF present in the media), the SV40 T antigen is expressed; this drives the cells to proliferate. However, upon withdrawal of the interferon and transfer of the cells to a higher temperature (39° C), T antigen expression is turned off and the cells stop proliferating and differentiate. Our studies have shown that differentiation is most pronounced when the dose of γ-INF used for stimulating proliferation at 33° C is 50 μg/ml.

Thus this model overcomes the difficulty of achieving stable integration of an exogenous oncogene into cellular DNA (Clement et al., 1990). Hence cells from the Immorto mouse can be driven to proliferate or differentiate in culture by turning the T antigen 'switch' on or off, with the addition or withdrawal of γ-INF, and by adjusting the temperature of the cultured cells. Because of these unique characteristics, Immorto mice have been successfully used to generate immortal cell lines of tissues that have hitherto proved difficult to culture such as colonic and small bowel epithelium (Whitehead et al., 1993) and bone (osteoclasts) (Chambers et al., 1993).

The T_7 cell line described in this paper was derived from a Type-II cell-rich fraction produced from lungs of H-2K^b-tsA58 (Immorto) mice using a dilution-cloning strategy. Morphologically, the T_7 cells closely resemble native Type-II cells. Specifically, the T_7 cells are polarized with distinct cytoplasmic lamellar bodies. The number and size of the lamellar bodies did appear to increase when the T_7 cells were grown under nonpermisssive conditions. In addition, there was an apparent increase in the number of lamellar bodies in the cells grown under nonpermissive conditions that were exposed to fresh media 4–6 h prior to harvesting. This observation suggests that the culture medium contains limiting quantities of the components required by the cells to produce lamellar bodies. The T_7 cells were also shown to synthesize SP-A, SP-B, SP-C, and phosphatidylcholine, the four major components of pulmonary surfactant.

Furthermore, the cells were demonstrated to secrete constitutively the fully or partially processed forms of all three surfactant proteins and phosphatidylcholine, indicating that the T_7 cells are able to manufacture and secrete surfactants. The T_7 cells demonstrate differentiated secretion of surfactant proteins and phospholipids at both 33 and 39° C. While the number of lamellar bodies increase at the nonpermissive temperature, the amounts of surfactant proteins exhibited by Western blot appear comparable. The most significant observed difference in the T_7 cell line at 39° C is the progressive loss of cell proliferation and eventual cell death, which do not occur at 33° C. Thus this line is the first, non–tumor-derived, immortalized and clonal Type-II cell line that appears to be able to differentiate and is capable of synthesizing and secreting surfactant. Unlike previously described tumor-derived Type-II cell lines, the T_7 cell line does not appear to have lost any Type-II cell specific biochemical or morphological features, and unlike primary Type-II cell cultures, T_7 cells can be maintained indefinitely in cell culture and clonally expanded with ease.

The unique Type-II cell line T_7 synthesizes both surfactant lipids and the proteins SP-A, SP-B, and SP-C. The lipids and all three surfactant proteins play an important role in the assembly of the surface-active lipoprotein complex, the lamellar body, and/or its extrusion from the cell. Recent ultrastructural and immunohistologic observations in the lungs of SP-B–deficient children and transgenic mice suggest that SP-B plays an important role in the assembly of the lamellar body, its intracellular trafficking, and in the metabolism of the surfactant proteins SP-A and SP-C (Nogee et al., 1993; deMello et al., 1994a, 1994b; Clark et al., 1995). Now, with the availability of a normal (non–tumor-derived) and clonal cell line that produces both surfactant lipids and proteins, these molecular mechanisms can be more clearly defined. The insight into Type-II cell biology that these studies will provide, could have a significant impact on the management of respiratory disorders that result from Type-II cell dysfunction, such as the adult and infantile respiratory distress syndromes.

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