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Proliferating cell nuclear antigen (PCNA/Cyclin) immunocytochemistry as a labeling index in mouse lung tissues

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Summary. Proliferating cell nuclear antigen is expressed in cells from late G_1 through the S-phase of the cell cycle. Therefore, antibodies directed against this molecule should provide a probe for labeling immunocytochemically the nuclei of proliferating cells. Herein we demonstrate the feasibility and reliability of this technique by quantifying immunostained pulmonary nuclei. We applied polyclonal and monoclonal antisera to alveolar and bronchiolar pulmonary epithelial cells in various proliferative states in tissue-sections and in vitro. A/J mice had a slightly higher labeling index than C57BL/6J mice, and proliferation in both strains increased dramatically after butylated hydroxytoluene treatment produced compensatory hyperplasia of Type-II pneumocytes. Immunostaining in fetal and neonatal lung samples from mice was higher than in adults. Spontaneous lung adenomas had a higher labeling index than the surrounding normal lung tissue. In addition, new data contained herein demonstrate a strain difference in proliferation of bronchiolar epithelial cells, and quantify the extent to which BHT-induced lung damage increases these proliferative rates. This mammalian nuclear antigen did not cross-react with antiserum to a functionally related bacterial protein, the beta subunit of *E. coli* DNA polymerase-III holoenzyme.

Key words: Cell proliferation – Immunocytochemistry – Lung – Bronchioles – Alveoli, lung – Proliferating cell nuclear antigen – Type II pneumocyte – Clara cell – Mouse (various strains)

Antibodies to proliferating cell nuclear antigen (PCNA) are among the various autoantibodies to nuclear antigens present in patients with systemic lupus erythematosus (SLE) (Miyachi et al. 1978; Takasaki et al. 1984). These antibodies have been used immunocytochemically to identify nuclei in proliferating cultured cell lines and frozen tissue-sections (Miyachi et al. 1978; Kurki et al. 1986, 1988). PCNA is synthesized late in G_1 and is especially abundant in S-phase nuclei (Bravo and Macdonald-Bravo 1985; Celis and Celis 1985; Kurki et al. 1986). It is present only in cells committed to DNA synthesis (Kurki et al. 1987). Cyclin, an acidic nuclear polypeptide (M_r 35000; pI 4.9) whose synthesis is also detectable only during late G_1 and S-phases (Bravo and Macdonald-Bravo 1985), has been shown by 2-D gel electrophoresis and immunoprecipitation to be identical to PCNA (Mathews et al. 1984). The auxiliary protein to DNA polymerase-delta which regulates DNA elongation is also identical to PCNA/cyclin, as shown by 2-D electrophoresis and immunoprecipitation, and by the fact that each stimulates SV40 DNA replication and increases the activity of DNA polymerase-delta (Prelich et al. 1987; Bravo et al. 1987). Tan et al. (1986) and Prelich et al. (1987) have suggested that PCNA and the beta subunit of *E. coli* DNA polymerase-III holoenzyme may be analogous since they have similar physical and functional properties. Antibodies to the beta subunit (if they bind PCNA selectively) could also be used to detect proliferating nuclei. We tested these molecules for immunologic cross-reactivity by using antiserum to the beta subunit and two anti-PCNA monoclonal antibodies (Mabs).

Because of our interest in cellular proliferation in mouse lung (Thaete et al. 1986), we asked whether PCNA immunostaining could be used as a reliable marker for dividing lung cells. PCNA was assayed under various conditions where cellular proliferation in the lung had been shown to vary according to other indices. These conditions included: (1) *Genetic variants.* A/J mice are prone to spontaneous neoplasia, while C57BL/6J (C57) mice are not (Andervont 1938). Using [^{3}H]thymidine autoradiography, we have previously demonstrated (Thaete et al. 1986) that the basal labeling index (LI) of alveolar Type-II cells in untreated adult A/J mice was significantly higher than in C57 mice. (2) *Acute lung injury.* Treatment of mice with a single injection of the food additive, butylated hydroxytoluene (BHT), causes extensive, but reversible, lung damage. Type-I cells die and are replaced following the proliferation and differentiation of Type-II cells (Adamson et al. 1977). During this repair process there is extensive compensatory hyperplasia of the Type-II cells (Saheb and Witschi 1975) and a milder hyperplasia of bronchiolar Clara cells (Smith et al. 1983). (3) *Neoplasia.* Lung neoplasms have a higher LI than their cell-type of origin (Dyson and Heppleston 1975). (4) *Normal lung development.* A peak of cellular division occurs in the parenchyma of mouse lung a few days after birth (Crocker et al. 1970).

An abstract of a portion of the work contained herein has been presented previously (Thaete et al. 1987).

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Materials and methods

Animals and treatment groups

Various inbred strains of mice were used for these studies to test the general applicability of PCNA immunocytochemistry. Strains A/J, C57BL/6J (C57), MA/MyJ, and BALB/cByJ (BALB) were obtained from The Jackson Laboratories (Bar Harbor, ME) and housed in accordance with guidelines from the National Institutes of Health. Normal adult male and female mice, 5–8 weeks old, were used. Mice were fed Wayne Lab Blox, given water ad libitum, and kept on a 12-h light-dark cycle. The animals were housed for at least 2 weeks before study.

The experimental groups for the BHT time-course consisted of A/J and C57 mice treated with either corn oil or with 400 mg BHT dissolved in corn oil/kg body wt. The lengths of time from treatment to analysis were 8 h, or 1, 4, 7, or 21 days. MA/MyJ mice with spontaneous lung adenomas, pregnant BALB mice from which fetal mice in mid-gestation (14–16 days) or late gestation (19–20 days) were obtained, and BALB neonates at 1, 5, or 14 days after birth composed the remaining groups. All groups included 2–4 mice.

Tissue preparation

The mice were sacrificed by cervical dislocation and their lungs infused intratracheally via a 19-gauge needle with 4% formaldehyde in 2% calcium acetate buffer. The lungs were removed and immersed for 4 h in the same fixative, and sliced into 1–2 mm sections for further processing. These fixed slices were rinsed for 2 h in running water and then incubated overnight in phosphate-buffered saline (PBS: 1.48 g Na_2HPO_4; 0.43 g KH_2PO_4; 7.2 g NaCl per liter; pH 7.2) containing 1 M sucrose. The samples were frozen in liquid nitrogen-cooled isopentane and stored in liquid nitrogen. Frozen tissues were mounted with OCT compound (Miles Labs., Naperville, IL) and sectioned on an IEC Minitome cryostat microtome at −20° C. They were mounted on albumin-coated glass slides and allowed to air-dry for 30 min before use.

Cell lines

PCC4, a rapidly proliferating line (mean doubling time in log phase = 26 h), established from a spontaneous mouse lung papillary tumor, was obtained from Dr. Stephen J. Kennel, Biology Division, Oak Ridge National Laboratory, and maintained in McCoy's 5A medium with 10% fetal calf serum (FCS), 100 IU penicillin, and 100 μg/ml streptomycin. A slower-growing cell line (doubling time = 48 h), NUL-1, established from a urethan-induced mouse lung alveolar adenoma, was obtained from Dr. Garry J. Smith, University of New South Wales, and maintained in CMRL 1066 medium with 10% FCS, 2.5 μg/ml Fungizone, 100 IU/ml penicillin, and 100 μg/ml streptomycin. PCNA immunostaining with Mabs was applied to these cells.

PCNA immunocytochemistry

Frozen sections. Tissue-sections were hydrated and rinsed in cold PBS (3 times for 5 min each); similar PBS rinses were interposed between all subsequent steps of the immunostaining procedure. Sections were then incubated with anti-PCNA (1:200), or normal (non-immune) human serum, in a moist chamber for 1 h at room temperature. The anti-PCNA (kindly provided by Dr. E.M. Tan, Scripps Clinic and Research Foundation, La Jolla, CA) had been isolated from serum of SLE patients and rendered monospecific to PCNA (Takasaki et al. 1984). Horseradish peroxidase-labeled goat anti-human IgG (1:200) was applied for 1 h followed by an incubation of 3–10 min in a substrate solution containing 0.025% 3,3′-diaminobenzidine in 50 mM Tris (pH 7.6) and 0.0025% H_2O_2 (Graham and Karnovsky 1966). 0.065% NaN_3 was added to the diaminobenzidine solution to inhibit endogenous peroxidase activity during development of the exogenous label (Tsutsumi et al. 1983). With each set of mouse tissues a positive control tissue (rat testis) was also stained. The duration of incubation of the slides in the diaminobenzidine solution was standardized by examination of the positive control slide. When the immature spermatogonia at the periphery of the testicular glands were discretely stained, all of the tissue-sections were removed from the diaminobenzidine solution. Sections were then rinsed in PBS and H_2O, and some were counterstained with methyl green to make all nuclei clearly visible, thus facilitating nuclear counting for the determination of LIs. All sections were dehydrated in a series of ethyl alcohol solutions of increasing concentration followed by two changes of xylene, and mounted in Permount for viewing, analysis, and photography.

Cultured cells. The methods of Ogata et al. (1987) were used except for the substitution of an enzyme label for the fluorescent label. Cells grown on coverslips to 70–80% confluence were quickly rinsed in PBS and fixed in freshly-prepared 1% paraformaldehyde in PBS (pH 7.2–7.4) for 1 min at room temperature. After a further rinse in PBS they were treated with 100% methanol for 10 min and then with 0.1% Nonidet P40 in PBS for 5 min, both at 0° C. The cells were rinsed in three changes of cold PBS and immunostained using a Histostain-SP biotin-streptavidin kit (Zymed, San Francisco, CA). Mabs $19A_2$ and $19F_4$ (Ogata et al. 1987; kindly provided by Dr. Eng M. Tan) were used at final dilutions of 1:100 and 1:50, respectively. Diaminobenzidine was the peroxidase substrate, as described above for use with frozen sections. Either PBS or antiserum to the beta subunit of *E. coli* DNA polymerase-III was used as a control primary antiserum in place of anti-PCNA.

Analysis

All immunostained sections were analyzed and scored in a blinded fashion without knowledge of the experimental group from which each lung sample was derived. Analysis of immunostained frozen sections was based on the number of labeled nuclei; the intensity of the stain was not taken into account since this varied between experiments. Labeled nuclei were counted at a magnification of 400x with the aid of an eyepiece grid reticle. At this magnification it was possible to differentiate cell types in the lung sections, as established previously (Thaete et al. 1986). Five fields of 100 alveolar cells for each of two sections from each animal were analyzed; LIs were thus based on 20–25 separate counts in each group. Bronchiolar LIs were determined by counting all nuclei in each bronchiolar profile in each sec-

tion examined. These bronchiolar counts were converted to percentages for comparison. The total number of bronchioles counted was approximately the same as the number of alveolar fields counted for each group. LIs were compared both among animals and among groups to identify statistically significant differences. The minimum acceptable level for the establishment of a statistically significant difference between mean values was $p<0.05$ in an analysis of variance. All quantitative results are reported as mean values $\pm$ SEM. The results of this analysis were compared with LIs reported in the literature for the various experimental groups.

Slot blotting

We tested PCNA and the beta subunit of *E. coli* DNA polymerase-III holoenzyme for antigenic similarity by slot-blot analysis. Purified beta subunit and polyclonal IgG directed against it (Johanson and McHenry 1980) were both provided by Dr. Charles McHenry (Univ. of Colorado Health Sciences Center, Denver, CO). Anti-PCNA Mabs were the same as those used for immunocytochemistry. Lungs from BHT-treated mice 4 days after treatment were homogenized in 6 volumes (wt/vol) of Tris-buffered glycerol and sucrose (TGS: 50 mM Tris HCl, pH 7.5, 20% glycerol, 1 mM EDTA, 5 mM dithiothreitol, 0.1 M NaCl, and 0.32 M sucrose) by 5 passes in a glass-teflon homogenizer. These homogenates were centrifuged at $600 \times g$ for 10 min. The nuclear protein-rich pellets were resuspended in 0.2 ml TGS, sonicated (6×5 sec), and then centrifuged at $10000 \times g$ for 30 min. The supernatants were loaded onto nitrocellulose (NC) in a Minifold II Slot-Blot System (Schleicher and Schuell, Keene, NH) by injecting 25 µl into each well on one side of the blotting apparatus. A solution of 5 µg purified beta subunit was loaded into adjacent wells.

The NC was cut into 3 sections, each containing 2 blots of beta subunit and 2 blots of lung homogenate nuclear fraction. Immunolabeling was done as follows: NC sections were incubated at room temperature for 1 h in a blocking solution (PBS with 0.05% Tween 20 and 5% dry skim milk), and then overnight at 4° C in a solution containing primary antibody. Each NC section was incubated with a different antibody: anti-beta at a dilution of 1:60, $19A_2$ at 1:1000, or $19F_4$ at 1:1000. All NC sections were washed in 3 changes of blocking solution for 10 min, and incubated at room temperature for 2 h in biotinylated anti-rabbit IgG for anti-beta primary serum, or biotinylated anti-mouse IgG (Vector Labs., Burlingame, CA) for the $19A_2$ and $19F_4$ mouse Mabs. After washing, the NC sections were incubated at room temperature for 1 h with a solution of streptavidin-horseradish peroxidase. NC was rinsed in 0.05 M Tris, pH 7.5 and immersed in a substrate solution (0.05 M Tris, 16.5% methanol, 0.049% 4-chloro-1-naphthol, and 0.0003% H_2O_2) until bands developed (3–5 min). After a brief rinse in distilled water, the NC blots were air-dried and photographed. Three separate slot-blot analyses were performed.

Results

Monospecific serum from SLE patients gave excellent results and was used for the frozen sections. The nuclei in many cells from formaldehyde-fixed, frozen lung tissue from mice stained with anti-PCNA (Figs. 1–6). No staining was observed in tissue-sections when normal human serum was substituted for anti-PCNA, demonstrating immunological specificity (Fig. 3, inset). Labeled cells have previously been shown mainly to include Type-II alveolar cells and only smaller numbers of other cell types (Thaete et al. 1986). A higher percentage of A/J Type-II pneumocytes exhibited stained nuclei than did C57 Type-II cells (Figs. 1, 2, and Table 1). Higher numbers of bronchiolar epithelial cells were stained in the lungs of untreated A/J mice compared with C57 (Table 1).

BHT treatment dramatically augmented the number of stained cells (Figs. 3, 4; Table 1), with a larger increase in A/J than C57 alveolar cells. The increased number of stained nuclei became evident after day 1 post-BHT. A peak of staining was maintained through days 4 and 7, and then staining returned to a control (untreated) level by 21 days post-BHT (data not shown). A post-BHT increase in stained bronchiolar nuclei was detected only in C57 mice.

Pulmonary adenomas which arose spontaneously in untreated MA/MyJ mice exhibited a higher number of stained nuclei (mean tumor LI = 48.3 ± 2.0) than the adjacent, non-neoplastic lung cells (Fig. 5). Both papillary adenomas (exhibiting a papillary arrangement of cells, pleomorphic nuclei, and a compressed tumor border), believed to arise from bronchiolar non-ciliated Clara cells (Kauffman et al. 1979),

Table 1. Comparison of the labeling indices for alveolar and bronchiolar epithelial cells labeled by PCNA immunocytochemistry in A/J and C57BL/6J mice[a]

Strain	Treatment	Alveolar	Bronchiolar
C57	None	8.1 ± 0.7[b]	21.9 ± 3.8[b]
A/J	None	23.1 ± 1.4[c]	37.7 ± 1.5
C57	4-Day BHT	51.4 ± 1.9	60.8 ± 1.5[d]
A/J	4-DAY BHT	57.9 ± 2.5	42.1 ± 3.3

[a] Labeled nuclei per 100 cells (mean $\pm$ SEM)
[b] Significantly less than untreated A/J and both BHT groups at $p<0.0001$
[c] Significantly less than BHT-treated A/J mice at $p<0.0001$
[d] Significantly greater than A/J 4-day post-BHT group at $p<0.0001$

Table 2. PCNA immunostaining in lung cells of fetal and neonatal BALB/cByJ mice[a]

	Mid-gestation	Late-gestation	1-day	5-day	14-day
Alveolar		28.3 ± 2.4	28.0 ± 1.1	51.6 ± 3.1[c]	33.5 ± 2.0
	36.6 ± 3.7[b]				
Bronchiolar		57.2 ± 2.6[d]	46.3 ± 2.9[e]	65.9 ± 2.2	64.8 ± 1.8

[a] Labeled nuclei per 100 cells (mean $\pm$ SEM)
[b] Alveoli and bronchioles could not be distinguished at mid-gestation
[c] Significantly greater than other alveolar groups at $p<0.001$
[d] Significantly greater than 1-day and less than 5-day and 14-day neonatal bronchiolar groups at $p<0.05$
[e] Significantly less than 5-day and 14-day neonatal bronchiolar groups at $p<0.001$

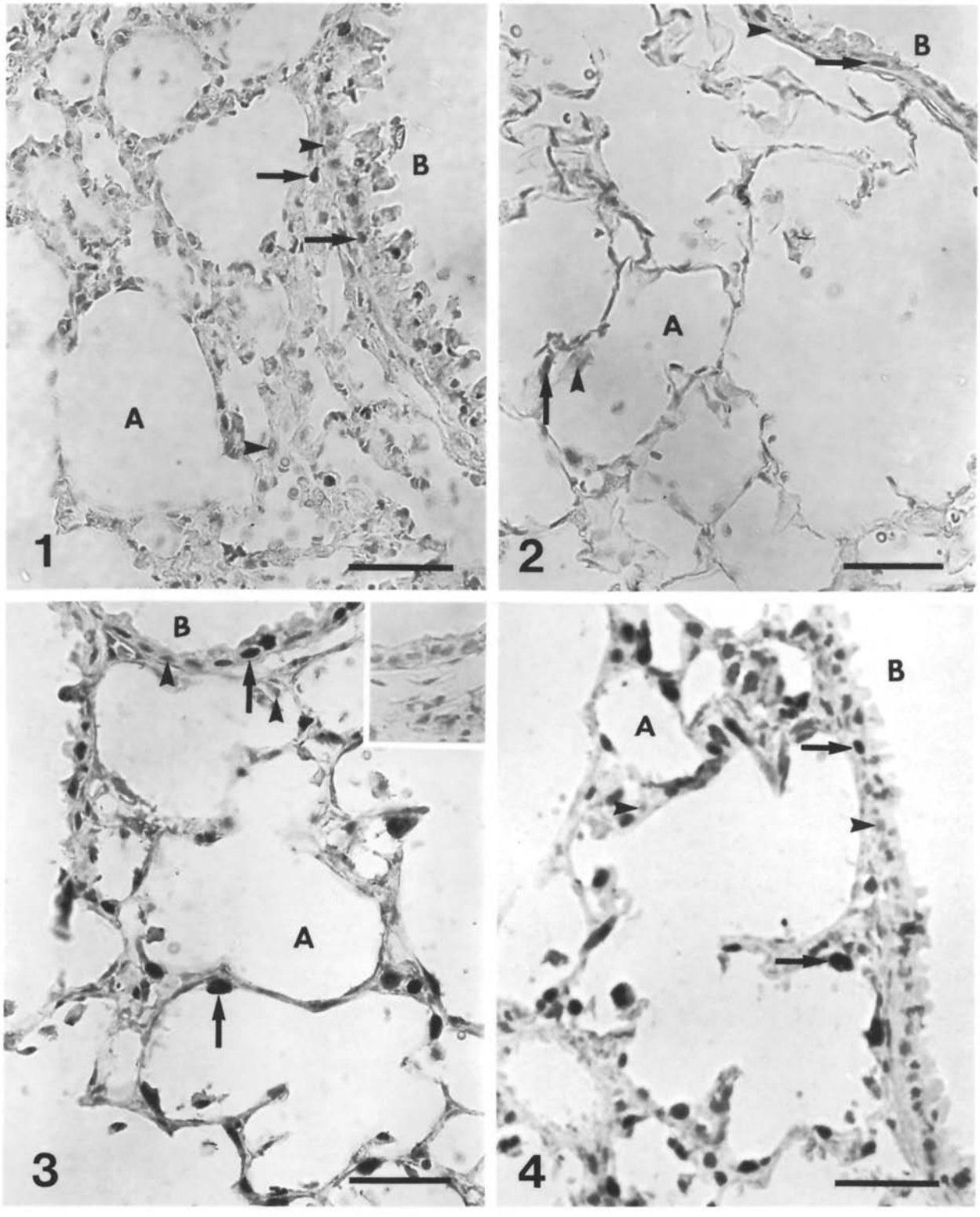

Fig. 1. Untreated strain A/J mouse lung immunostained for PCNA. Some nuclei (*arrows*) in both bronchiolar (*B*) and alveolar (*A*) epithelia are stained. Methyl green used as nuclear counterstain, but photomicrograph taken using green filter to show contrast between stained and unstained nuclei (*arrowheads*). This lung was collapsed during fixation and hence appears to exhibit higher degree of cellularity compared with Fig. 2. × 350; Bar = 50 μm

Fig. 2. Untreated C57 mouse lung immunostained for PCNA. Few alveolar cells (*A*) or bronchiolar cells (*B*) are stained (*arrows*) in this view, corresponding with the very low LI determined for untreated C57 lungs. Several unstained nuclei (*arrowheads*) are visible throughout this micrograph. × 350; Bar = 50 μm

Fig. 3. Strain A/J mouse lung immunostained for PCNA 4 days after treatment with BHT. Many bronchiolar (*B*) and alveolar (*A*) cells are stained (*arrows*) during peak of hyperplastic response to BHT toxicity. Methyl green nuclear counterstain was used for determination of LIs, but section photographed using green filter to show absence of peroxidase label in unstained nuclei (*arrowheads*). *Inset:* Adjacent lung section carried through immunostaining procedure, but with normal human serum applied instead of anti-PCNA. Lack of immunolabeling in any nuclei is evident. × 350; Bar = 50 μm

Fig. 4. Lung section (*A* alveolus; *B* bronchiole) from BHT-treated C57 mouse, immunostained for PCNA 4 days after treatment. Hyperplastic response and PCNA staining are maximal. Examples of stained (*arrows*) and unstained (*arrowheads*) nuclei are present in photomicrograph. × 350; Bar = 50 μm

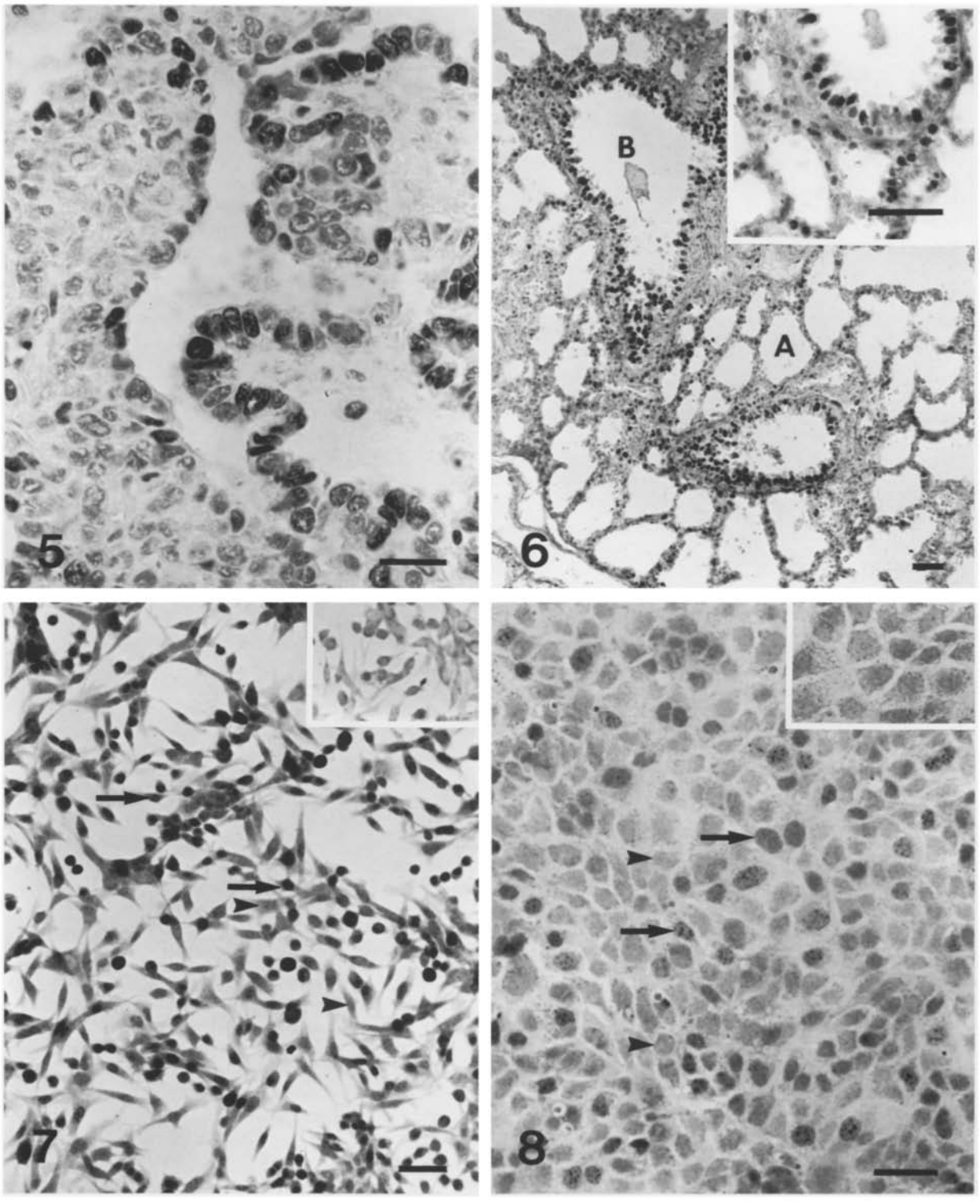

Fig. 5. Spontaneous papillary adenoma from MA/My mouse lung. PCNA immunostaining reveals many proliferating cells within tumor. ×450; Bar = 25 μm

Fig. 6. PCNA immunostaining in 5-day-old BALB mouse. Stained nuclei in bronchiolar cells (*B*) are especially prominent, while alveolar cells (*A*) exhibit more lightly-stained nuclei. ×100; Bar = 50 μm. *Inset:* Additional detail of bronchiolar staining. ×250; Bar = 50 μm

Fig. 7. PCC4 cells grown on glass coverslip and immunostained with anti-PCNA Mab $19A_2$. Most nuclei in this rapidly-growing cell line are labeled (*arrows*) and stand in sharp contrast to unlabeled nuclei (*arrowheads*). *Inset:* Immunostaining of these PCC4 cells, using PBS in place of the primary antibody $19A_2$, yielded no nuclear staining, demonstrating specificity of Mabs. ×160; Bar = 50 μm

Fig. 8. NUL-1 cells near confluence on glass coverslip and stained with anti-PCNA Mab $19F_4$. Fewer nuclei are labeled (*arrows*) in this slower-growing cell line than in PCC4 cells (*Fig. 7*). Note punctate staining in some nuclei and diffuse staining in others, while nuclei in cells which are apparently not proliferating are completely unstained (*arrowheads*). *Inset:* NUL-1 cells immunostained using antiserum to beta subunit of *E. coli* DNA polymerase-III holoenzyme. Although background is slightly higher, no cells stained in this procedure, demonstrating specificity of antibody $19F_4$ and absence of beta subunit cross-reactivity in these cells. ×200; Bar = 50 μm

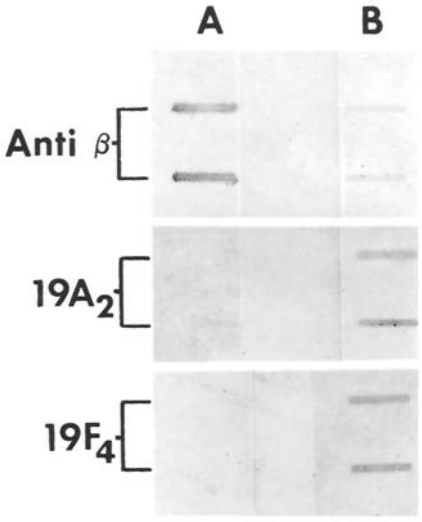

Fig. 9 A, B. Slot-blot of purified beta subunit of *E. coli* DNA polymerase-III holoenzyme and nuclear fraction from homogenates of mouse lung. These were immunostained with anti-beta subunit or with Mab $19A_2$ or $19F_4$ as indicated. Each well in column **A** was loaded with 5 μg purified beta subunit, and each well in column **B** was loaded with 25 μl of mouse lung nuclear extract. Anti-beta subunit reacted with purified beta subunit, but not with the mouse lung nuclear fraction. Slight densities in column B in anti-beta section are from residual hemoglobin and were of same intensity both before and after immunostaining. Mabs $19A_2$ and $19F_4$ both reacted with mouse lung nuclear fractions, but not with beta subunit. This figure demonstrates specificity of antisera as well as complete lack of cross-reactivity between beta subunit and anti-PCNA, and between lung nuclear proteins and anti-beta

and alveolar adenomas (with a compact cellular arrangement, rounded nuclei, and indistinct tumor borders), believed to arise from alveolar Type-II cells (Grady and Stewart 1940; Svoboda 1962), were examined. There was no significant difference in the LIs of the two tumor types.

Developing lung cells in mid-gestational BALB fetuses had many stained nuclei (Table 2). A large number occurred in the bronchiolar epithelium through late gestation, decreased slightly in 1-day-old neonatal mice, and then increased at 5 days after birth (Fig. 6). Alveolar epithelial cells were also highly labeled at 5 days postpartum, and staining was maintained at nearly twice the adult level through day 14 postpartum (Tables 1, 2).

Cultured cells immunostained with Mab $19A_2$ or $19F_4$ exhibited many labeled nuclei (Figs. 7, 8). Some nuclei were labeled homogeneously while others had a punctate staining pattern. The faster-growing PCC4 cell-line (Fig. 7) contained more labeled nuclei in a given area than did the more slowly-growing NUL-1 line (Fig. 8). Cells treated with PBS showed no staining (Fig. 7, inset). Attempts to stain cells with anti-beta subunit yielded completely negative results (Fig. 8, inset).

In the slot-blot analysis (Fig. 9) antibody to *E. coli* DNA polymerase III beta subunit reacted with purified beta subunit, but did not label pulmonary nuclear fractions. Conversely, anti-PCNA Mabs $19A_2$ and $19F_4$ labeled nuclear extracts of lung, but did not cross-react with purified beta subunit.

Discussion

PCNA immunocytochemistry provides a reliable index of cell proliferation in lung tissue of the mouse. Changes in the degree of PCNA staining reflected the known changes in proliferative activity of the lung that occur as a result of BHT treatment, during normal fetal and neonatal development, and in neoplasia. PCNA immunostaining corresponds well with published autoradiographic LIs. For example, our previous autoradiographic results (Thaete et al. 1986) disclosed a 2–3-fold higher LI in Type-II cells of untreated A/J lungs than in untreated C57 lungs, a difference that is also evident using PCNA immunostaining. Autoradiographic data using untreated control lung tissue of mice (Thaete et al. 1986) were obtained on alveolar cells, but not on bronchiolar cells. In the present study, however, nonciliated Clara cells, the only bronchiolar epithelial cells which proliferate (Evans et al. 1978), were stainable and quantifiable by PCNA immunostaining. These results illustrate the greater sensitivity of the PCNA method. This is the first study to show that proliferation of bronchiolar Clara cells is different in two mouse strains, which also exhibit a significant difference in proliferative rates of Type-II cells.

Four days after BHT treatment, the alveolar cell LI is 3–6 times higher than in untreated mice. Adamson et al. (1977) observed this same level of increased LI by [^{3}H]thymidine autoradiography after BHT treatment. The increased proliferation of bronchiolar epithelial cells (Fig. 4, Table 1) following BHT treatment has not been quantified previously. Neonatal mouse lungs have been shown by Crocker et al. (1970) to have a very high [^{3}H]thymidine LI compared with adult animals. Correspondingly, both the alveolar and bronchiolar LIs, determined by PCNA immunocytochemistry for fetal and neonatal mice, were among the highest LIs detected. The high LIs observed with pulmonary adenomas correspond with published autoradiographic data for neoplastic tissues (Dyson and Heppleston 1975; Thaete et al. 1986).

Ahnen et al. (1987) have shown that PCNA immunostaining in rat colon provides similar information about the rates and localization of proliferation as that obtained by [^{3}H]thymidine autoradiography, ornithine decarboxylase activity, and flow cytometry. PCNA immunostaining gave a reproducibly higher LI compared with autoradiography. This is as expected since detection of radioactive decay emissions depends on their being trapped by a photographic emulsion in a single plane above the section, whereas immunocytochemistry labels molecules without regard to their orientation in the tissue section. Also, PCNA is detectable in late G_1, throughout S-phase (Celis and Celis 1985), and in very early G_2 (Kurki et al. 1986), while a short pulse of [^{3}H]thymidine labels the cell only during S-phase.

PCNA immunostaining varies in intensity from day to day, and the procedure must be standardized against a positive control tissue. In spite of this disadvantage, the method gives reproducible results. Autoradiography is tedious to perform and quantify. PCNA immunostaining is more rapid, simpler, and provides a clear discrimination even at low magnifications, which makes quantitation faster. Radioactive materials can be avoided with this procedure, which is also a notable advantage. Celis and Celis (1985) described a highly discrete intranuclear localization of PCNA during S-phase. The diffuse, nucleolar, and ultimate-

ly punctate localizations may be useful in differentiating sub-phases of this cycle, which is not possible to do with autoradiography.

Tan et al. (1986) and Prelich et al. (1987) noted the physical and functional similarities of PCNA and the beta subunit of *E. coli* DNA polymerase-III holoenzyme. If these two molecules were antigenically similar and if PCNA were recognized by the antibodies to the beta subunit, then anti-beta subunit could provide another immunocytochemical tool for detecting proliferating cells. Our results with the slot-blot analysis, using one polyclonal and two Mabs, imply a lack of antigenic homology between these 2 proteins, however. This suggests that the eukaryotic and prokaryotic molecules are likely to be distinct proteins functioning in similar roles.

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