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Binding of [^{3}H]Benzo(*a*)pyrene to DNA in Cultured Human Bronchus[1]

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SUMMARY

The studies reported here demonstrate some of the factors affecting the binding of benzo(*a*)pyrene (BP) to macromolecules in cultured human bronchial mucosa. Bronchial specimens were obtained at either surgery or "immediate" autopsy from patients with and without lung cancer. Grossly normal-appearing pieces of bronchus were cultured in a chemically defined medium, *i.e.*, CMRL 1066 medium containing 1 μg insulin per ml, 0.1 μg β-retinyl acetate per ml, 0.1 μg hydrocortisone hemisuccinate per ml, 2 mM L-glutamine, 100 units penicillin G per ml, and 100 μg streptomycin per ml. After 7 days, explant cultures were exposed to [^{3}H]BP, usually for 24 hr, and then binding to total cellular macromolecules was studied by autoradiography, and binding to DNA was measured following isolation of DNA from bronchial mucosal cells. The extent of binding of [^{3}H]BP was dependent on dose of BP, length of exposure to [^{3}H]BP, and temperature. By autoradiography, bronchial epithelial cells bound more [^{3}H]BP than stromal fibroblasts. Both 7,8-benzoflavone and butylated hydroxytoluene appeared to reduce the level of [^{3}H]BP bound to DNA, while nicotine apparently did not alter the level of binding. These studies demonstrate that the bronchial mucosa, an important human cancer target tissue, has the capability to form metabolites of BP which bind to macromolecules including DNA. In addition, 7,8-benzoflavone and butylated hydroxytoluene, both known to alter the microsomal metabolism of BP, reduce the level of [^{3}H]BP bound to DNA.

INTRODUCTION

The extrapolation to man of carcinogenesis data from studies utilizing experimental animals presents several complex problems. One approach to provide a link between these experimental studies and human cancer is the development of experimental systems to study carcinogenesis in important human target tissues. One facet of our attempt to establish such a system has been to investigate the binding of known and suspected carcinogens to macromolecules in cultured human bronchus. We have previously reported that cultured human bronchial mucosal cells can bind carcinogenic PAH[6] to their macromolecules (11). Carcinogenic PAH were chosen for these initial metabolic studies because they are found in tobacco smoke and the urban atmosphere, both of which have been implicated in the etiology of bronchogenic carcinoma (10, 24, 38, 45). Studies reported here concern the further analysis of the parameters, *e.g.*, time, temperature, and dose, which may alter the binding of BP by cultured bronchial mucosa. In addition, selected compounds found to inhibit the metabolism of BP in cells of experimental animals were examined to determine whether they altered the level of [^{3}H]BP bound to DNA in human bronchial mucosa.

Binding of [^{3}H]BP was measured by both a biochemical and an ARG assay, these assays complementing one another. ARG allows the cellular localization of [^{3}H]BP within the bronchial mucosa which contains several types of cells with various states of differentiation. Binding of [^{3}H]BP determined by ARG estimates binding to the total cellular macromolecules. In contrast, the biochemical assay used in these studies measures the total binding of [^{3}H]BP to a specific macromolecule, DNA, without consideration of differences between individual epithelial cell species. Metabolic activation of [^{3}H]BP into chemically active forms capable of binding to cellular macromolecules is presumably an important facet of the binding determined by both assays.

MATERIALS AND METHODS

Bronchial specimens were obtained at either surgery or immediate autopsy (36) from 11 patients with and without lung cancer (Table 1). Grossly normal-appearing pieces of bronchus, mostly from segmental bronchi, were immediately immersed in Medium L-15 (22) at 4° until cultured.

Explant Culture Conditions. Bronchi were cut into approximately 1- × 1-cm squares and placed in 60-mm plastic Petri dishes with the epithelium oriented toward the gas-liquid interface (34). The number of bronchial pieces obtained from a single case ranged from 10 to 96. Two ml of CMRL 1066 medium containing 1 μg crystalline bovine insulin per ml (Eli Lilly, Indianapolis, Ind.), 0.1 μg hydrocorti-

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[6] The abbreviations used are: PAH, polynuclear aromatic hydrocarbons; BP, benzo(*a*)pyrene; ARG, autoradiography; DMSO, dimethyl sulfoxide; BHT, butylated hydroxytoluene; AHH, aryl hydrocarbon hydroxylase; BF, 7,8-benzoflavone.

Table 1
Source of human bronchi

Case identification no.	Age (yr)	Sex	Diagnosis	Obtainment
3	58	M	SCC[a]	S
22	58	M	SCC	S
25	63	M	AC	S
26	20	M	HI	A
30	53	M	AC	S
36	56	F	MC	S
39	46	F	AC	S
41	48	M	AC	S
43	56	M	OCC	S
44	60	M	AC	S
46	56	M	LCC	S

[a] SCC, squamous cell carcinoma of the lung; S, surgery; AC, adenocarcinoma of the lung; HI, head injury; A, immediate autopsy (36); MC, metastatic renal carcinoma to the lung; OCC, oat cell carcinoma; LCC, large cell carcinoma of the lung.

sone hemisuccinate per ml (Upjohn Co., Kalamazoo, Mich.), 0.1 μg β-retinyl acetate per ml (Hoffmann LaRoche, Nutley, N. J.), 100 units penicillin G per ml, 100 μg streptomycin per ml, and 2 mM L-glutamine were added to each dish. Cultures were maintained in an air-tight box at 36.5° in an atmosphere of 50% O_2, 45% N_2, and 5% CO_2. The medium was replaced and the atmosphere of the cultures was reestablished by flushing the box with the gas mixture at 4 liters/min for 5 min every 2 days. The box containing the cultures was rocked in the dark at approximately 10 cycles/min so that the bronchial tissue was submerged one-half of the time.

Incubation with [^{3}H]BP or [^{14}C]BP. The radiopurity of [^{3}H]BP (25 Ci/mmole; Amersham/Searle Corp, Arlington Heights, Ill.) and [^{14}C]BP (18.3 mCi/mmole; Amersham/Searle) was determined by radioscan of thin-layer chromatograms, and when necessary it was repurified by thin-layer chromatography (16) to greater than 99%. On the 7th day of explant culture, either [^{3}H]BP or [^{14}C]BP was dissolved in DMSO (final concentration was usually 0.5%; DMSO used in these experiments was redistilled and stored under nitrogen; Pierce Chemical Co., Rockford, Ill.) and added to the culture medium, generally at a final concentration of either 1.5 μM ([^{3}H]BP) or 65 μM ([^{14}C]BP) for 24 hr.

In selected experiments, either BF (20 μM; Aldrich Chemical Co., Milwaukee, Wis.), BHT (2 or 10 μM; Sigma Chemical Co., St. Louis, Mo.), or nicotine *dl*-bitartrate (0.1, 1, or 10 μM; Sigma) were also dissolved in DMSO and added to the medium. Following incubation, the bronchial tissues were rinsed in 3 washes of cold Dulbecco's phosphate-buffered saline.

Isolation of [^{3}H]BP or [^{14}C]BP Bound to DNA. The bronchial mucosa containing epithelial and mesenchymal cells was scraped from the supporting structures into Dulbecco's phosphate-buffered saline. Mucosal cells from 4 to 6 cultures were pooled for each experimental variable. One to 4 cultures for each experimental variable were also fixed for examination by light microscopy and/or ARG. The cell suspension was transferred to a small Potter-Elvehjem homogenizer, the suspension was adjusted to 5 mM trisodium EDTA and 2% sodium dodecyl sulfate, and the mixture was homogenized with 20 strokes at 0°.

The homogenate was treated at room temperature with an equal volume of water-saturated phenol. After centrifugation, the aqueous phase and the protein pellicle at the interface were removed and saved. Two volumes of ethanol were added to this mixture, which was then stored at −20° overnight. The resulting precipitate was sedimented by centrifugation at 10,000 × *g* for 2 min, the supernatant was discarded, and the precipitate was dissolved in 0.5 ml of 0.15 M NaCl in 0.1 M Tris-HCl, pH 7.5. This aqueous solution was extracted twice with equal volumes of ether to remove residual phenol. Any residual ether was evaporated in a stream of nitrogen. This aqueous solution was incubated with Pronase (Calbiochem, LaJolla, Calif.; final concentration 100 μg/ml; previously incubated at 80° for 10 min) for 1 hr at 37°. Unbound [^{3}H]BP was then further extracted with ether until the ether solutions were free of radioactivity by liquid scintillation counting (16).

The aqueous solution was then sequentially digested with pancreatic RNase (Worthington Biochemical Corp., Freehold, N. J.; final concentration 100 μg/ml; previously incubated at 95° for 5 min to inactivate DNase) at 37° for 1 hr and with Pronase (100 μg/ml) at 37° for 1 hr. After enzymatic digestion, the samples were dialyzed at 4° against multiple changes of 0.15 M NaCl-0.1 M Tris-HCl, pH 7.5, during a 3-day interval. After dialysis, the sample solutions were adjusted to 5 M CsCl and 4.2 mM trisodium EDTA, and the samples were centrifuged at 35,000 rpm for 66 hr at 24° in a Beckman SW 56 rotor. After centrifugation, the CsCl gradients were fractionated into 0.2-ml portions with a gradient fractionator while the absorbance of the effluent stream was continuously monitored at 254 nm. The absorbance of peak fractions was determined at 260 and 280 nm, and the radioactivity in all fractions was determined by scintillation counting.

ARG of Bound [^{3}H]BP. The location of bound [^{3}H]BP was determined by quantitative light microscopic ARG (3, 12). In brief, bronchial pieces (4/experimental variable) were fixed in 2.7% glutaraldehyde buffered by 0.1 M *s*-collidine, pH 7.4, at 4° overnight, the pieces were postfixed in 1.3% OsO_4 in the same buffer for 2 hr. All tissue-dehydrating solutions were monitored for radioactivity by liquid scintillation counting. The tissues were repeatedly washed with each solution until unbound radioactivity was removed, before being treated with the next dehydrating solution (12). Increasing concentrations of ethanol (50, 75, 95, and 100%) and then 2 changes of 100% propylene oxide at room temperature were used for dehydration. The 1- x 1-cm pieces of bronchi were embedded in a mixture of Epon-Araldite (12). One-μm sections were placed on glass slides also containing 1-μm sections of poly([^{3}H]butyl)methacrylate (18 μCi/g; Amersham/Searle) which serve as internal standards (3). The slides were then dipped in Eastman Kodak NTB-2 emulsion (Eastman Kodak Co., Rochester, N. Y.) at 45°, dried, and stored in total darkness at 4° with desiccant for 2, 5, and 14 days. The autoradiograms were developed at 18° in D19 for 5 min, rinsed in distilled water, fixed in Kodak Rapid Fixer for 10 min, and rinsed twice in distilled water for 30 min. The sections were then stained with toluidine blue (35) at room temperature for 1 to 2 hr. Cells and ARG grains were counted as previously described (12).

RESULTS

Our initial efforts were directed at defining the factors influencing binding of [^{3}H]BP in cultured human bronchial mucosa. To determine the variability of the measurements of [^{3}H]BP binding between tissue samples within a case, [^{3}H]BP was added to 12 bronchial cultures obtained from 1 case. After incubation for 24 hr, the 12 cultures were divided into 3 equal groups, and DNA was obtained from the bronchial mucosa of each group. The variation of the samples due to experimental procedure was less than 10% (Table 2). Biological variation among individuals is currently being studied, and these data will be reported elsewhere.

The extent of binding of [^{3}H]BP was dependent on dose of [^{3}H]BP and temperature. It appeared to be dependent also on length of exposure. Increasing levels of [^{3}H]BP bound to DNA were found with increasing doses of [^{3}H]BP in the range of concentrations examined (Table 3). Kendall's rank order test for trend was used to test the hypothesis of no relationship between binding and dose against the hypothesis of increased binding with increasing dose (33). The analysis, using data from Experiments 1 and 2, showed that the observed upward trend of binding with dose is significant ($p < 0.05$). To investigate the effect of temperature on binding of [^{3}H]BP, bronchial tissues were incubated at either 0° or 36.5°. Observations were made on 4 cell types at the 2 temperatures (Table 4; Figs. 1 and 2). The binding of [^{3}H]BP to cellular macromolecules was reduced at 0° ($p < 0.01$). Furthermore, the analysis showed a significant difference ($p < 0.01$) in binding by cell type, which may, in part, reflect differences in the cross-sectional area of these cells. In comparison to epithelial cells, fibroblasts bound significantly less [^{3}H]BP ($p < 0.01$) on a cellular basis (Table 4).

Table 2

Variation in [^{3}H]BP bound to DNA caused by experimental methods

Seven-day-old organ culture incubated for 24 hr in a chemically defined medium containing [^{3}H]BP (1.5 μM; 25 Ci/mmole). Final concentration of DMSO was 0.5%. Tissue was obtained from Case 46 (see Table 1).

Sample	[^{3}H]BP bound to DNA[a] (dpm/μg DNA)
1	105
2	119
3	122

[a] Mean ± S.D. of the 3 values was 115.3 ± 9.1.

Table 3

Effect of [^{3}H]BP dose on its binding to DNA in cultured human bronchial mucosa

Seven-day-old organ culture of human bronchus incubated for 24 hr in chemically defined medium containing [^{3}H]BP (25 Ci/mmole). Final concentration of DMSO was 0.5%. Tissue was obtained from Case 43 in Experiment 1 and from Case 44 in Experiment 2 (see Table 1).

	[^{3}H]BP bound to DNA (pmoles/mg DNA)	
[^{3}H]BP (μM)	Experiment 1	Experiment 2
0.15	1.4	1.8
1.50	17.0	22.9
15.00	92.0	105.0

Table 4

Effect of incubation temperature binding of [^{3}H]BP by cultured human bronchial mucosal cells

Seven-day-old organ cultures of human bronchus were incubated at either 0° or 36.5° for 3 hr in chemically defined medium containing [^{3}H]BP (1.5 μM; 25 Ci/mmole). Tissue was obtained from Case 3 (see Table 1). Autoradiograms were exposed for 14 days and prepared as previously described (3).

	No. of autoradiographic grains with incubation temperature of	
Cell type	0°	36.5°[a]
Basal		
Nucleus	1.1 ± 0.3[b]	6.5 ± 0.7
Cytoplasm	1.2 ± 0.2	5.1 ± 0.6
Total cell	2.3 ± 0.4	11.5 ± 1.0
Mucous		
Nucleus	1.3 ± 0.3	6.9 ± 0.6
Cytoplasm	2.4 ± 0.3	6.8 ± 0.5
Total cell	3.7 ± 0.5	13.7 ± 0.7
Ciliated		
Nucleus	1.4 ± 0.3	10.0 ± 0.9
Cytoplasm	2.0 ± 0.4	11.1 ± 0.7
Total cell	3.3 ± 0.5	21.1 ± 1.2
Mesenchymal fibroblast		
Nucleus	0.4 ± 0.2	2.0 ± 0.3
Cytoplasm	0.4 ± 0.2	1.6 ± 0.3
Total cell	0.8 ± 0.3	3.6 ± 0.4

[a] All 36.5° values are significantly greater than the 0° values ($p < 0.01$). A 2-way analysis of variance, after taking the square root of the ARG counts, was done. The square root transformation was used because a preliminary examination of the count data revealed that the variation was roughly proportional to the mean of the count.

[b] Number of autoradiographic grains (mean ± S.E.) within nuclear or cytoplasmic compartment of cell (to the nearest one-tenth). Fifty cells of each cell type were counted for each experimental variable.

Table 5

Effect of exposure time on [^{3}H]BP binding to DNA in cultured human bronchial mucosa

Seven-day-old organ culture of human bronchus incubated for either 4, 12, or 24 hr in chemically defined medium containing [^{3}H]BP (1.5 μM; 25 Ci/mmole). Final concentration of DMSO was 0.5%. Tissue was obtained from Case 22 (see Table 1).

Exposure time (hr)	[^{3}H]BP bound to DNA (dpm/μg DNA)
4	114
12	178
24	618

The decrease in binding of [^{3}H]BP from 36.5° to 0° was not uniform by cell type. The ciliated cells showed the greatest decrease (significantly greater than the other cells at $p < 0.01$) and the mesenchymal fibroblasts showed the smallest decrease ($p < 0.01$) when compared to the other cell types. Focal areas of hyperplasia and metaplasia in the bronchial epithelium also contained tightly bound [^{3}H]BP (Fig. 3). For length of exposure to [^{3}H]BP, the data are consistent, with an increase in binding with increased time of exposure (Table 5; Figs 4 to 6). The value at 24 hr is markedly greater than those at 4 and 12 hr. Tritium instead of ^{14}C was used as

a radioactive label of BP in most of these studies because the specific activity of [^{3}H]BP available from commercial sources is approximately 1000-fold greater than that of [^{14}C]BP. Since some of the radioactivity measured could be due to exchange of tritium, [^{14}C]BP was used in 1 experiment. The level of [^{14}C]BP bound to DNA was 591 pmoles/mg DNA. The high level of binding may be caused by the concentration of [^{14}C]BP (65 μM) used in this experiment, 50-fold greater than the usual concentration of [^{3}H]BP (1.5 μM), which may have markedly induced the activity of AHH. Additional studies determining the relationship among binding of BP to DNA and activity of AAH are needed.

A 2nd set of experiments was done to investigate the effect of the addition of BF, BHT, or nicotine to the incubation medium on binding of [^{3}H]BP to DNA. Previous studies (13, 31, 42, 44) had suggested that these compounds might alter BP metabolism and binding to DNA. In these experiments, both BF and BHT reduced the observed level of [^{3}H]BP bound to DNA (Tables 6 and 7), while nicotine apparently did not alter the level of binding (Table 8).

The morphology of the bronchial mucosa cultured in this chemically defined medium is described in the preceding report (1). The addition of BP at concentrations ranging from 0.15 to 15 μM for 24 hr did not cause significant morphological lesions observed by light microscopy. Occasional cells containing large lysosomes were found at the highest dose of BP tested (15 μM) for 24 hr.

DISCUSSION

A number of tissues and cell types in both experimental animals and humans can metabolize carcinogenic PAH. While the detailed steps in the metabolism of BP are still being defined, evidence of AHH activity has been found in several human cells and tissues, including placenta (26, 28, 43), skin fibroblasts (23), cultured embryonic cells (4, 14), liver (18, 28, 29), pulmonary macrophages (6), blood monocytes (2, 30), blood leukocytes (5), and blood lymphocytes (6, 17, 44). We have previously reported investigations (11) demonstrating that cultured human bronchial epithelium can presumably metabolize carcinogenic PAH. 7,12-Dimethylbenz[*a*]anthracene, 3-methylcholanthrene, BP, and dibenz[*a,h*]anthracene were metabolized into forms that tightly bind to cellular macromolecules including DNA. The studies reported here demonstrate some of the factors affecting the binding of BP to macromolecules in human bronchial mucosa. As expected, the binding of [^{3}H]BP was altered by those factors that influence activation of [^{3}H]BP by microsomal enzymes, such as temperature (Table 5) and concentration of [^{3}H]BP (Table 3). Time of exposure to [^{3}H]BP also appeared to alter the extent of binding (Table 4).

A variety of synthetic and naturally occurring compounds are known to alter the metabolism of PAH (13, 31, 39–42, 44) and, in some cases, inhibit their tumorigenicity in experimental animals (37, 39, 40). Such inhibition may eventually provide one means of intervention to prevent human lung cancer (10). Selected chemicals are being studied in the present *in vitro* system employing the human bronchial mucosa. BF has been shown to inhibit AHH in a variety of animal cells and, in cultured human lymphocytes, 100 μM BF reduces the AHH activity to 50% of the control level (44). In our study with a 5-fold lower concentration of BF (20 μM), binding of [^{3}H]BP to DNA in human bronchial mucosa was reduced to 36% of the control level (Table 6). Since the quantitative relationship between activity of AHH and BP bound to DNA has not been established, it is impossible to make meaningful comparisons. There is also a need to measure simultaneously both activity of AHH and binding levels of BP to DNA in the target cells, *e.g.*, bronchial epithelial cells, as well as a more accessible cell population such as blood cells, to determine whether a positive correlation exists among these different cell populations. Selkirk *et al.* (31) found that BF uniformly inhibits the formation of each of the BP metabolites and suggested that this lack of selective inhibition indicates that BF acts on either the oxidase(s) or a prior component of the microsomal electron chain.

The metabolism of BP by the microsomal oxygenase system requires oxygen and NADPH. Antioxidants inhibit metabolism of BP in microsomal fractions (13) and can reduce chromosomal breakage caused by PAH (32). Although exceptions exist (9), they frequently inhibit the tumorigenicity of chemical carcinogens in experimental animals (37, 39–41). The effect of antioxidants, commonly added to preserve food, and of selenium and vitamin E in the diet on carcinogenesis in man is unknown. BHT, a common food additive, appears to inhibit the level of [^{3}H]BP bound to DNA in cultured human bronchial mucosa (Table 7), and this inhibition may be dependent on the dose of BHT.

Nicotine and BP are constituents of tobacco smoke. Weber *et al.* (42) have shown that nicotine inhibits the metabolism of BP in rat lung both *in vivo* and *in vitro*. Prior treat-

Table 6

Inhibition of [^{3}H]BP binding to DNA in cultured human bronchial mucosa by BF

Seven-day-old organ cultures incubated for 24 hr in medium containing BF and [^{3}H]BP (1.5 μM; 25 Ci/mmole). Final concentration of DMSO was 0.5%. For Experiment 1, tissue was obtained from Case 25 and, for Experiment 2, tissue was obtained from Case 26 (see Table 1).

	[^{3}H]BP bound to DNA (dpm/μg DNA)	
BF (μM)	Experiment 1	Experiment 2
None	139	183
20	39	81

Table 7

Inhibition of [^{3}H]BP binding to DNA in cultured human bronchial mucosa by BHT

Seven-day-old organ cultures incubated for 24 hr in medium containing BHT and [^{3}H]BP (1.5 μM; 25 Ci/mmole). Final concentration of DMSO was 1%. For Experiment 1, tissue was obtained from Case 36 and, for Experiment 2, tissue was obtained from Case 30 (see Table 1).

	[^{3}H]BP bound to DNA (dpm/μg DNA)	
BHT (μM)	Experiment 1	Experiment 2
None	246	304
2	52	NT[a]
10	35	118

[a] NT, not tested.

Table 8

Effect of nicotine on [³H]BP binding to DNA in cultured human bronchial mucosa

Seven-day-old organ culture incubated for 24 hr in chemically defined medium containing nicotine hydrogen tartrate and [³H]BP (1.5 μM; 25 Ci/mmole). Final concentration of DMSO was 0.5%. For Experiment 1, tissue was obtained from Case 30 and, for Experiment 2, tissue was obtained from Case 39 (see Table 1).

	[³H]BP bound to DNA (dpm/μg DNA)	
Nicotine (μM)	Experiment 1	Experiment 2
0.0	113	132
0.1	NT[a]	117
1.0	NT	128
10.0	108	141

[a] NT, not tested.

ment of rats with nicotine decreased the activity of AHH in homogenates of rat lung. The addition of nicotine (1, 10, and 100 μM) directly to homogenates of lung inhibited activity of AHH by 16, 18, and 35%, respectively. Under the experimental conditions tested here, nicotine did not alter the level of [³H]BP bound to DNA in human bronchial mucosa (Table 8). Comparison is made difficult because different cell populations have been studied, *i.e.*, homogenates of whole lung may not reflect the metabolic properties of the target cells of respiratory carcinogens, the bronchial epithelial cells. Biochemical and morphological methods have been adopted for studying respiratory epithelial cells in an animal model of lung cancer (12, 15, 16), but extrapolation among species still remains difficult. Finally, the metabolic pathway of BP metabolism might conceivably differ among species and among tissues within the same species. These complexities suggest the need to couple investigations using human cells with concurrent studies using the appropriate experimental animal cells.

PAH are toxic only to those cells that metabolize them. Crocker *et al.* (7) have shown that a high dose of BP (55 μM) is markedly cytotoxic to human bronchi cultured on plasma clots for 2 weeks. Lasnitzki (20, 21) observed hyperplasia and squamous metaplasia in cultures of human fetal lung exposed to PAH. The respiratory epithelium in organ cultures from experimental animals has also undergone metaplastic changes induced by PAH (8, 19, 25, 27). In the short-term studies reported here, occasional cells containing large lysosomes were found by light microscopy in the cultured bronchial mucosa exposed for 24 hr to the highest does of BP (15 μM). Studies to determine the long-term effects of carcinogenic PAH on cultured human bronchial epithelium are in progress.

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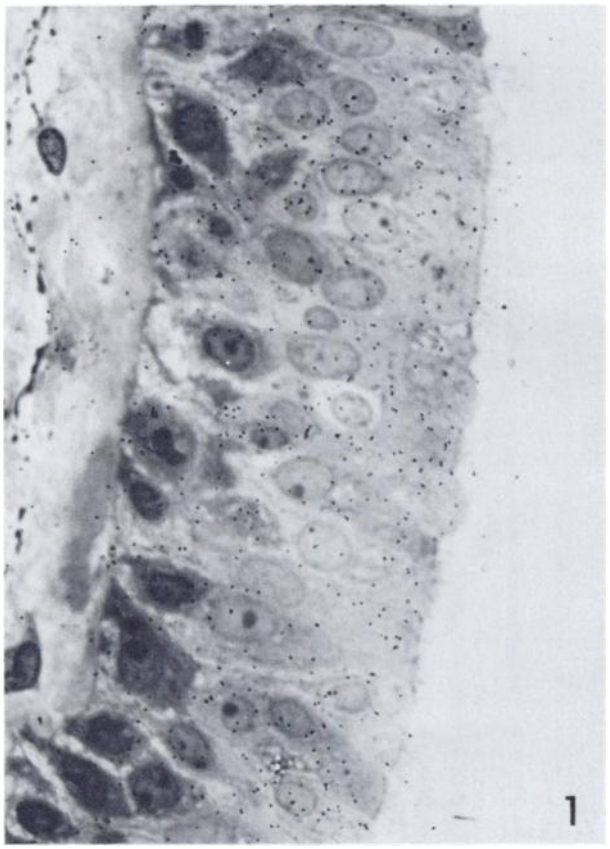

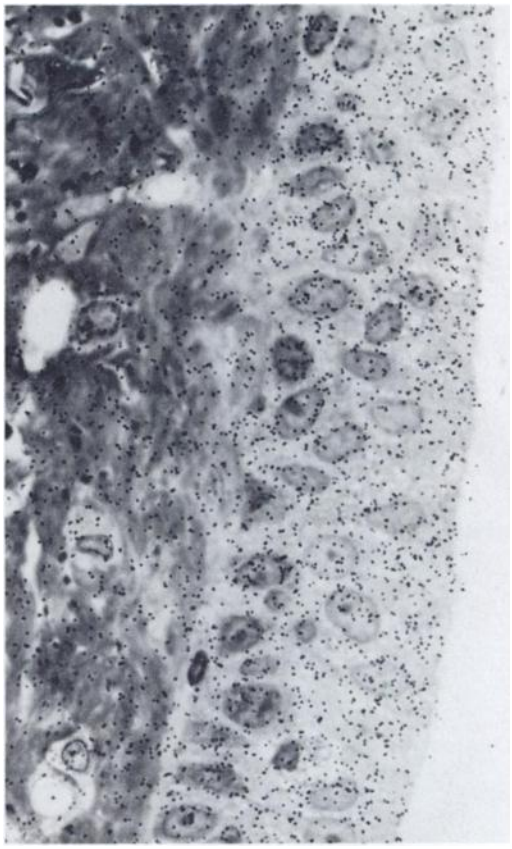

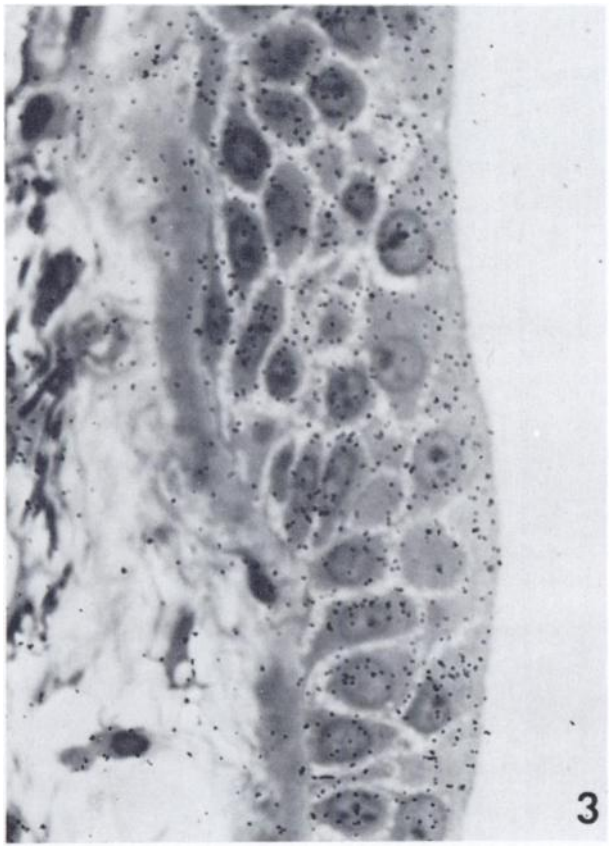

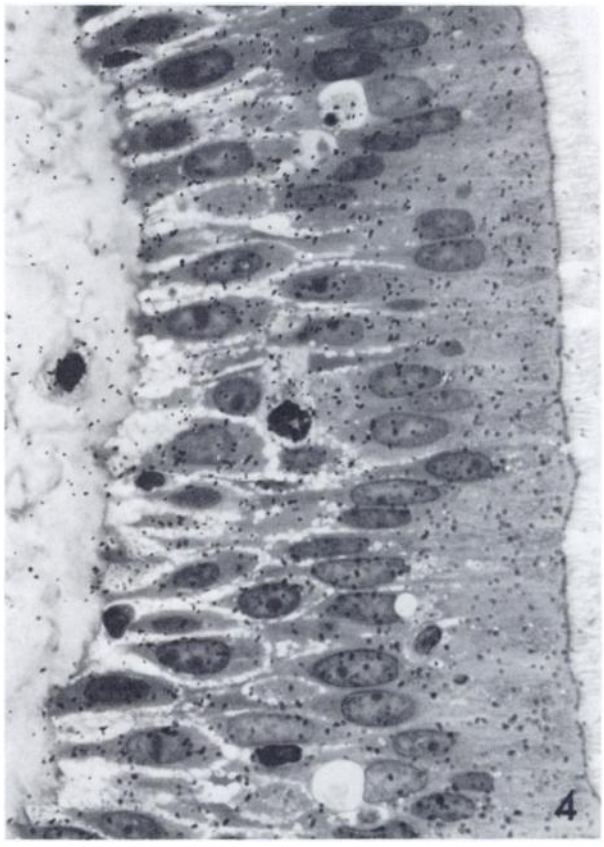

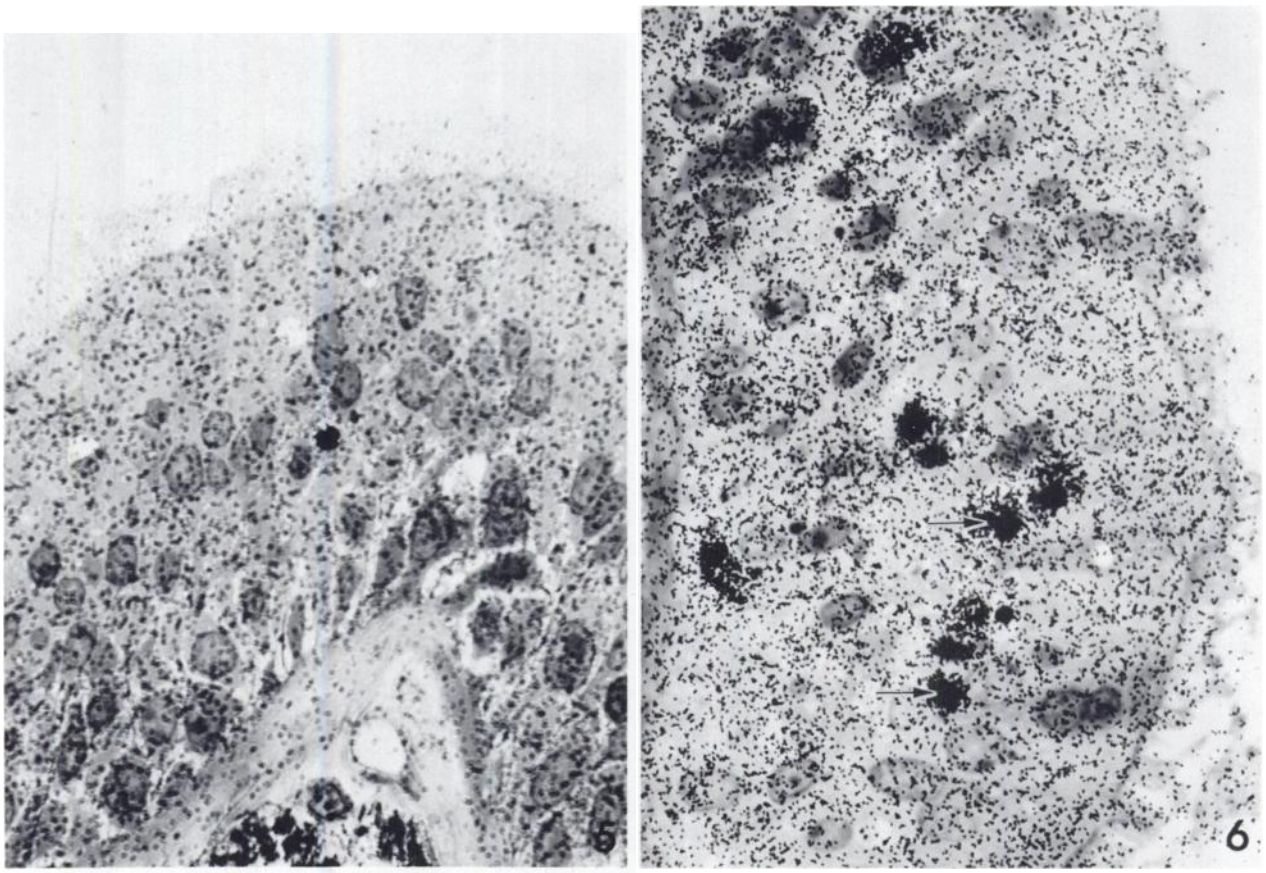

Figs. 1 to 6. Autoradiograms of human bronchial epithelium cultured for 7 days prior to the addition of [^{3}H]BP (1.5 μM). Toluidine blue, ×880.
Fig. 1. Binding of [^{3}H]BP to cultured human bronchial epithelium incubated at 0° for 3 hr.
Fig. 2. Binding of [^{3}H]BP to cultured human bronchial epithelium incubated at 36.5° for 3 hr.
Fig. 3. Binding of [^{3}H]BP to metaplastic cultured human bronchial epithelium incubated for 4 hr.
Fig. 4. Binding of [^{3}H]BP to cultured human bronchial epithelium incubated for 4 hr.
Fig. 5. Binding of [^{3}H]BP to cultured human bronchial epithelium incubated for 12 hr.
Fig. 6. Clusters of ARG grains are occasionally seen (*arrows*). Binding of [^{3}H]BP to cultured human bronchial epithelium incubated for 24 hr.