Bleomycin-Induced Injury and Metaplasia of Alveolar Type 2 Cells

Relationship of Cellular Responses to Drug Presence in the Lung

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Metaplastic epithelial cells are often observed lining alveoli in bleomycin-induced pulmonary fibrosis. The hypothesis that these cellular changes are induced by the direct action of the drug on differentiating Type 2 cells is now examined in a sequential study to correlate the presence of ^3H bleomycin in the lung with the pattern of injury and repair of the alveolar epithelium. A single intravenous dose or multiple small intraperitoneal doses induce focal necrosis of Type 1 epithelial cells followed by Type 2 cell regeneration. At the time of maximal deoxyribonucleic acid (DNA) synthesis in these cells, significant amounts of ^3H bleomycin are demonstrable in the lung by scintillation counting; and in autoradiographs, the drug appears to concentrate in epithelial cells. Subsequently many abnormal Type 2 cells are seen. Some are binucleate, and others show nuclear disruption. The usual process of differentiation to Type 1 cells does not occur; instead, a variety of epithelial forms are found, including fetal-like tubular structures and ciliated and squamous metaplastic cells. The correlation of epithelial injury and repair with the direct demonstration of bleomycin in the lung indicates that Type 2 cells are susceptible to injury in the division and differentiation phases of the cell cycle and may then produce a variety of inappropriate alveolar lining cells. (Am J Pathol 96:531–544, 1979)

THE ADMINISTRATION of the antineoplastic drug bleomycin to human beings or to experimental animals is associated with injury to the air–blood barrier and the development of pulmonary fibrosis.[1,2] Whereas repair of the alveolar epithelium is usually accomplished by division of Type 2 cells, with subsequent transformation to the Type 1 form,[3] recovery after bleomycin is characterized by epithelial metaplasia.[4] It has been postulated that the Type 2 cell, generally considered to be resistant to injury, may be susceptible to the cytotoxic drug bleomycin during the cycle of division and differentiation that follows injury to Type 1 cells.[4] Presumably this abnormal reparative process depends upon the presence of the drug in the lung at the critical time of Type 2 cell division. This hypothesis is now tested by injecting tritiated bleomycin to mice to correlate the level of drug in the lung with the sequential patterns of injury and repair of the alveolar epithelium.

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Supported by Medical Research Council of Canada Grant MT3878.

Accepted for publication March 20, 1979.

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0002-9440/79/0809-0531$01.00

Materials and Methods

Group 1

One hundred Swiss albino mice (25-g males, Charles River CD1 strain) were given a single intravenous injection of 120 mg/kg bleomycin. Four animals were killed by intraperitoneal injection of sodium pentobarbital at Days 0, 1, 2, 3, 5, 7, 10, 12, 14, 16, and 21. One hour before death, each mouse received 2 μCi/g tritiated thymidine intraperitoneally. Following a tracheotomy, the lungs were expanded with 4% phosphate-buffered glutaraldehyde, and a small sample was postfixed in osmic acid and embedded in Spurr resin for electron microscopy. The bulk of the tissue was postfixed in formalin and subsequently embedded in glycol methacrylate. Sections less than 1 μ thick were stained by hematoxylin and eosin for histologic examination. Autoradiographs were prepared by dipping sections in Kodak NTB2 emulsion, exposing them for 2 weeks, developing and staining with toluidine blue. The number of thymidine-labeled nuclei was determined at each time interval by counting 5000 cells, excluding the bronchi, on each mouse. The methacrylate sections are thin enough to allow identification of the labeled cells. At each time 400 labeled cells were identified and the differential percentages of the various cell types in the lung were determined.

Group 2

Bleomycin was tritiated commercially and diluted with "cold" bleomycin such that a dose of 20 μCi/g was administered (specific activity 0.2 mCi/mg). Fifty mice received a single intravenous injection of ^{3}H bleomycin (120 mg/kg) and were killed in sets of 3, at the above times, with the addition of one set at 12 hours. After tracheotomy, two samples of lung, liver, and kidney, about 30 mg each, were removed, washed thoroughly in saline, and incubated overnight at 55 C in a tissue solubilizer prior to scintillation counting. The results were corrected for background and calculated as disintegrations per minute (dpm). Mean values were plotted against time after injection. Samples of lung were prepared for histologic and autoradiographic examinations in an attempt to identify the cellular location of the tritium label.

Group 3

Fifty mice received 20 mg/kg tritiated bleomycin by intraperitoneal injection twice weekly for 8 weeks. Three animals were killed at biweekly intervals to 20 weeks after the initial injection. Tissue was prepared for scintillation counting, light and electron microscopy as detailed above. In all studies, animals killed at zero time served as controls.

Results

Intravenous Injection

The morphologic sequence of lung changes in Groups 1 and 2 were similar, indicating that the process of tritiation did not alter the pulmonary toxicity of bleomycin. The sequence of early changes that has already been reported [2,4] will be briefly described. Endothelial-cell swelling and necrosis was observed between Days 2 and 5 (Figure 1A). Injury to Type 1 epithelial cells occurred between Days 7 and 10, but no changes were observed in Type 2 cells at this time. The Type 1 cells became edematous and often necrotic, leaving a denuded interstitium that facilitated the passage of a fibrinous exudate to the alveoli (Figure 1B). Starting

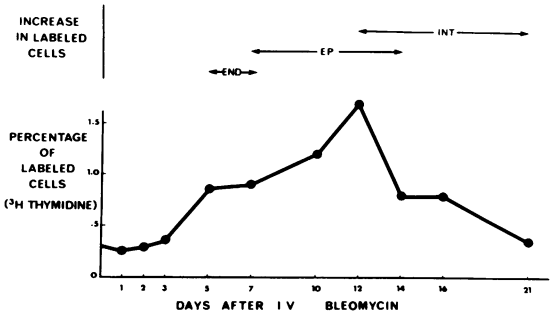
at 10 days after injection, there was proliferation of Type 2 epithelial cells, and many alveoli became lined by these cuboidal cells, which incorporated ^{3}H thymidine (Figure 2). At 3 weeks many giant Type 2 cells were observed, sometimes almost filling an alveolus (Figure 3A); occasional ciliated cells were seen on the alveolar surface (Figure 3B). These cells were not in continuity with the bronchioles, which showed no evidence of injury or increased thymidine incorporation.

Synthesis of deoxyribonucleic acid (DNA) in alveolar cells is shown in Text-figure 1. In the first few days after bleomycin injection, the percentage of labeled cells did not exceed the low control values. Increased DNA synthesis in the lung, observed from Day 5 onward, peaked 12 days after bleomycin. Differential cell counts revealed three sequential, though overlapping, components to this regenerative response: 1) Endothelial labeling rose from 18 ± 2% in controls to 28 ± 4% at Day 5. 2) Epithelial labeling rose from 12 ± 2% in controls to 20 ± 3% from Days 10 to 16 (Figure 2). 3) Interstitial cell labeling rose from 30 ± 3% to 40 ± 5% from Day 12 onward.

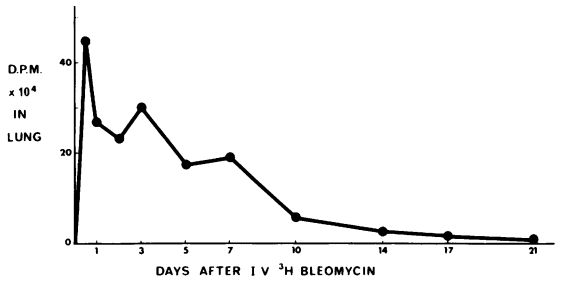
Scintillation counting showed a high level of ^{3}H bleomycin in the lung within 1 day, and a measurable amount (10,000 dpm) was still present 2 weeks after a single injection (Text-figure 2). From calculations using specific activity this corresponds approximately to 1 μg of bleomycin in the lungs. Levels in kidney and liver, about three times as high initially, were very low after 2 weeks. In the autoradiographs after ^{3}H bleomycin, silver grains were seen at the endothelium of small vessels and capillaries up to 5 days after injection (Figure 4). Subsequently the label was low and diffuse over the lung tissue, and no site of predominant label could be determined.

Intraperitoneal Injections

The initial stages of cellular injury following multiple doses of bleomycin, though delayed, resembled those induced by a large intravenous



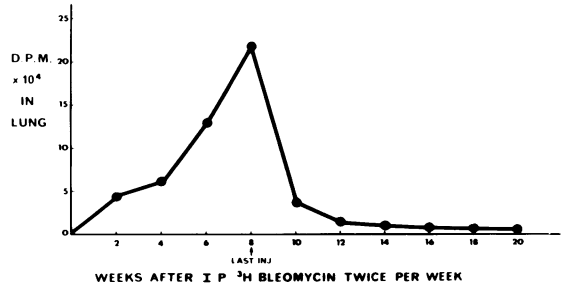
TEXT-FIGURE 1—*Lower*—Percentage of ^{3}H thymidine-labeled cells after a single intravenous injection of bleomycin (120 mg/kg). *Upper*—Pulmonary cell types showing increased thymidine incorporation determined from differential counts of labeled cells. END = endothelial; EP = epithelial; INT = interstitial.



TEXT-FIGURE 2—Mean values for radioactivity (dpm) in 30-mg lung samples at intervals after a single intravenous injection of ^3H bleomycin (120 mg/kg).

dose; endothelial damage was seen after 2 weeks, and Type 1 cell necrosis from 4 weeks onward. After 6 weeks some cuboidal epithelial cells were seen in mitosis, and small tubules of epithelial cells, similar to those shown in Figure 2, were observed. From 8 weeks onward alveolar epithelial cells showed a variety of forms. Many giant Type 2 cells were seen, they were often binucleate, and disruption of nuclear structure occurred in some cells (Figures 5A, B, and C). Subsequently, mitotic activity continued in the epithelium without the usual transformation to Type 1 cells. The persisting cuboidal alveolar cells assumed a number of different forms. Tubules of bizarre-shaped cells with prominent nucleoli were seen (Figure 6A); ciliated cells were formed and occasional foci of squamous metaplasia lined the original alveoli (Figure 6B).

Scintillation counting showed a steady buildup of ^3H bleomycin in the lung over the course of the injections (Text-figure 3). After 8 weeks, when epithelial cell division and metaplasia were well developed, radioactivity in the lung was 20×10^4 dpm; the values in liver and kidney were somewhat higher but dropped sharply when drug administration stopped. At the peak of incorporation, the distribution of silver grains in the autoradiographs was generally diffuse; however, some unusually large epithelial cells exhibited several grains, mainly over the nuclei (Figure 7).



TEXT-FIGURE 3—Mean values for radioactivity (dpm) in 30-mg lung samples at intervals after twice-a-week intraperitoneal injections of ^3H bleomycin (20 mg/kg) for 8 weeks.

Discussion

The attenuated endothelial and Type 1 epithelial cells of the air–blood barrier are particularly susceptible to injury by a variety of blood-borne or airborne agents. Acute injury at these sites by agents such as oxygen or nitrogen dioxide results in endothelial injury with rapid regeneration, followed by necrosis of Type 1 cells.[3,5] Epithelial repair is accomplished by division of Type 2 cells, some of which transform to the Type 1 form to restore the normal air–blood barrier.[3] A similar process occurs in the lung after chemical injury induced by butylated hydroxytoluene.[6,7] The initial events in bleomycin toxicity are similar, with injured endothelial cells undergoing rapid regeneration. This suggests that bleomycin, though injuring these cells, is not selectively bound to their nucleic acids. The subsequent regenerative response of the epithelium is altered; Type 1 cell necrosis is followed by Type 2 cell division. However, the usual process of differentiation to Type 1 cells does not occur. Instead, a variety of epithelial forms are seen, including fetal-like tubular structures, ciliated cells, and squamous metaplastic cells. The overall sequence of cellular injury and repair reveals that epithelial injury and regeneration occur before the fibroblastic response, indicating that metaplasia is not a result of an initial change in the pulmonary interstitium. It is more likely that the inappropriate differentiation is related to the binding of bleomycin to nucleic acid of epithelial cells, and in some instances severe nuclear damage to Type 2 cells is observed.

In the steady state, the Type 2 cell, with its slow turnover, is generally considered to be injury resistant. However, in the regenerative phase the total population of these cells may turn over in 3 days.[3] The results of the present experiment suggest that Type 2 cells are susceptible to injury in the proliferative phase of the cell cycle. The therapeutic action of bleomycin is dependent on its ability to control the division of tumor cells, and it is particularly effective on tumors of epithelial origin.[8] The susceptibility of proliferating Type 2 cells would depend upon the presence of the toxic agent at the time of cell division. Two methods have been used to measure bleomycin in tissue. The original antibacterial assay used by Umezawa [9] has been superseded by the more sensitive measurement of the retention of radiolabeled drug.[10] Both methods show that the drug is retained in skin and lung for several days after injection.[9,10] The present study was designed to correlate the presence of the drug in the lung with the sites of cellular injury. After a single intravenous injection, significant levels of bleomycin are present during the periods of Type 1 cell necrosis and Type 2 cell proliferation. It is suggested that the subsequent metaplasia is due to the continuing presence of the drug in Type 2 cells at the critical phase

of mitosis. This hypothesis is supported by the results of the intraperitoneal experiments in which the administration of additional small doses at the time of Type 2 cell proliferation induced exaggerated epithelial metaplasia. At this time progressive accumulation of ^{3}H bleomycin is observed by scintillation counting, and the autoradiographs indicate a selective concentration of the drug in Type 2 cells. It is concluded that there is sufficient bleomycin present to affect the Type 2 cell during its proliferative phase. Division of these cells occurs, but it appears that the processes of cellular differentiation are disturbed.

The susceptibility of epidermal cells to bleomycin is thought to be related to low levels of detoxifying enzymes that cleave the carboxyamide groups of the drug.[11] Once in the nucleus, bleomycin inserts itself between the double helix, causing strand scission.[12] This process is reversible; however, if the cell is involved in replication before DNA is repaired, an abnormal cell is produced,[13] leading to secondary changes in RNA and protein.[14] *In vitro* experiments have shown that the differential susceptibility of cells to bleomycin depends on the phase of the cell cycle.[15] Some cells may be inhibited from entering mitosis; others, with DNA damage during G_1 and S phases, may replicate abnormally. In mouse skin, bleomycin decreases the number of cells in mitosis and their passage time through the cycle and inhibits cellular maturation.[16] The effects on epithelial differentiation have been demonstrated in a clinical study on carcinoma of the tongue, in which bleomycin treatment reduced cell division and promoted the accumulation of tonofilaments in tumor cells, which later produced keratinic epithelial pearls.[17] From the results of the present study, it is suggested that replication of Type 2 cells occurs in the presence of bleomycin but the effects of the drug on DNA subvert the subsequent processes of differentiation, to produce a variety of inappropriate alveolar lining cells.

The relationship of bleomycin to epithelial metaplasia and pulmonary fibrosis may have relevance in clinical situations where fibrosing alveolitis is encountered after drug treatment.[1,18] Whereas acute injury to endothelium and epithelium may be rapidly repaired with no fibrosis,[3,19] continuing injury, with delayed or disturbed regeneration, is associated with fibroblastic activity.[20,21] Type 1 cells are particularly vulnerable to injury, and it is usually assumed that the Type 2 cell is injury-resistant. This assumption should now be reassessed in light of the demonstrated susceptibility of this cell during division, at which time persisting or newly administered cytotoxic agents may cause injury. This is of particular significance in chemotherapy where the repeated, frequent administration of drugs such as bleomycin increases the chance of drug interaction with

Type 2 cells dividing in response to previous injury to Type 1 cells. The disordered epithelial repair that results disturbs the usual interrelationships between epithelial and fibroblastic cells. It is suggested that this change may be a factor in the development of pulmonary fibrosis.

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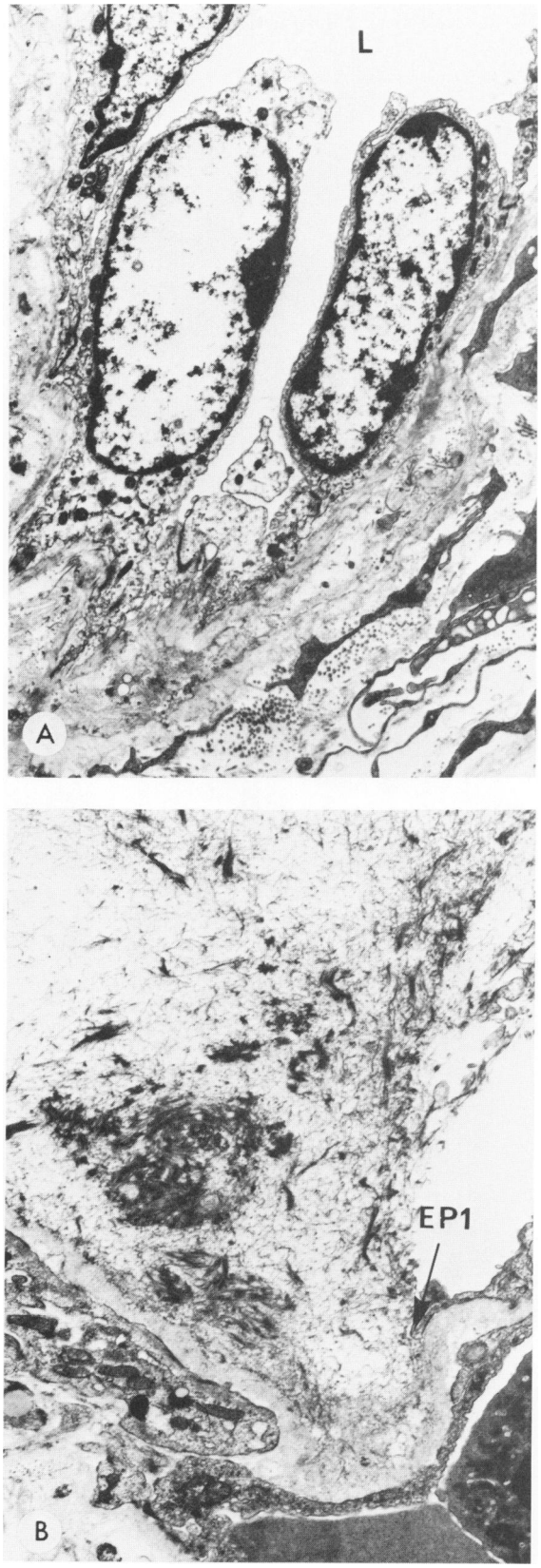
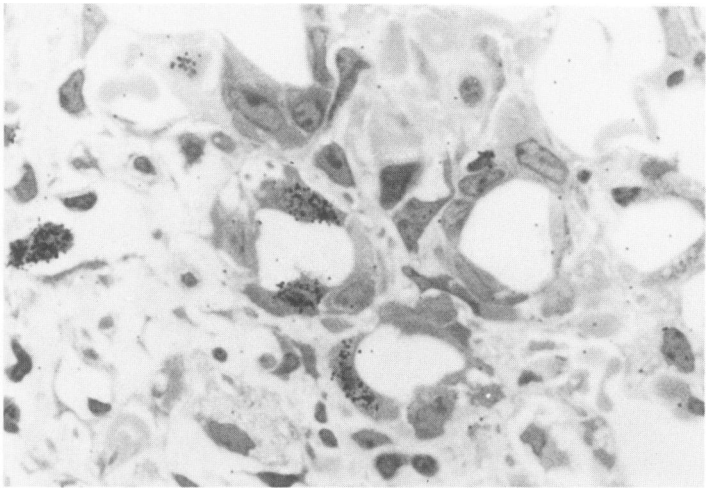


Figure 1A—Lining the vascular lumen (*L*), endothelial cells show edematous cytoplasm with nuclear lysis, 3 days after intravenous bleomycin. (×5000) **B**—Focal necrosis of Type 1 cells (*EP1*) with a fibrinous exudate in the alveolus, 7 days after intravenous bleomycin. (×8500) (With a photographic reduction of 7%)



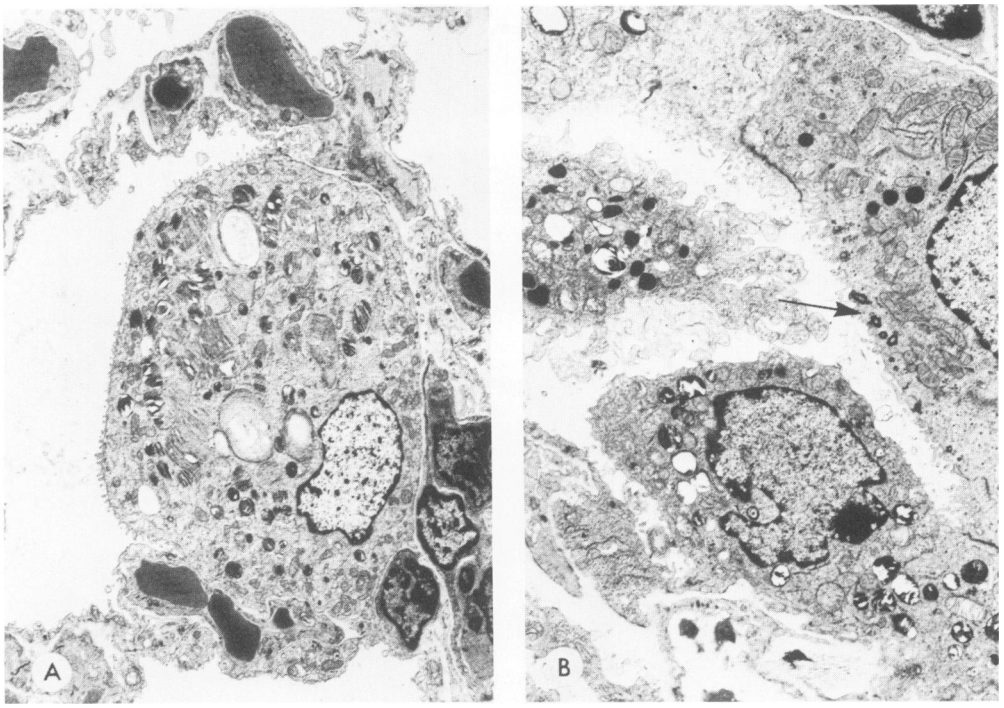


Figure 2—Autoradiograph 12 days after intravenous bleomycin, ^{3}H thymidine 1 hour before death. Tubules of epithelial cells, many in DNA synthesis, line the alveoli. (×960) **Figure 3**—Three weeks after intravenous bleomycin. **A**—Giant Type 2 cell almost fills an alveolus. (×2500) **B**—Type 2 cells and a cell with ciliary basal bodies (*arrow*) and microvilli line the alveolus. (×3500) (Both with a photographic reduction of 19%)

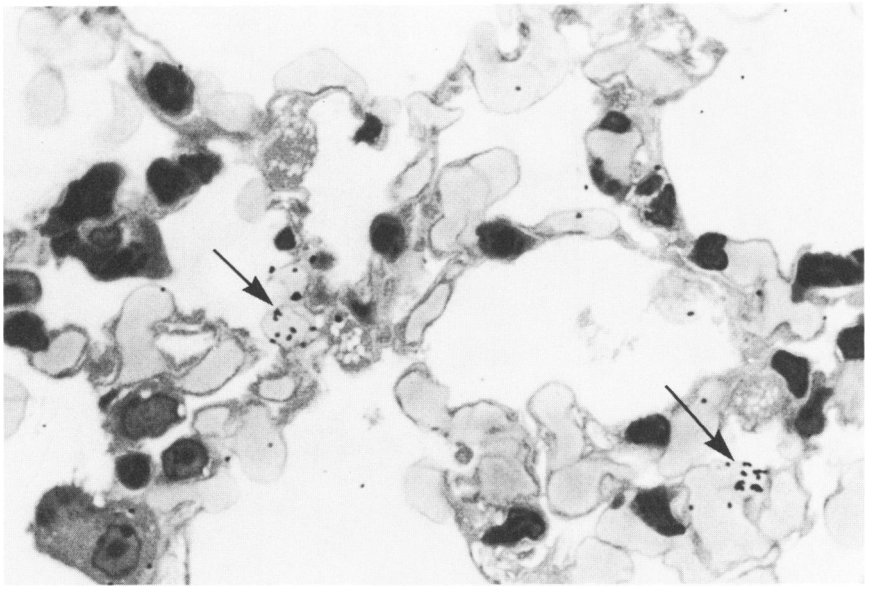


Figure 4—Autoradiograph, 3 days after intravenous ^{3}H bleomycin, shows concentration of silver grains (*arrows*) at the capillary endothelium. (×960)

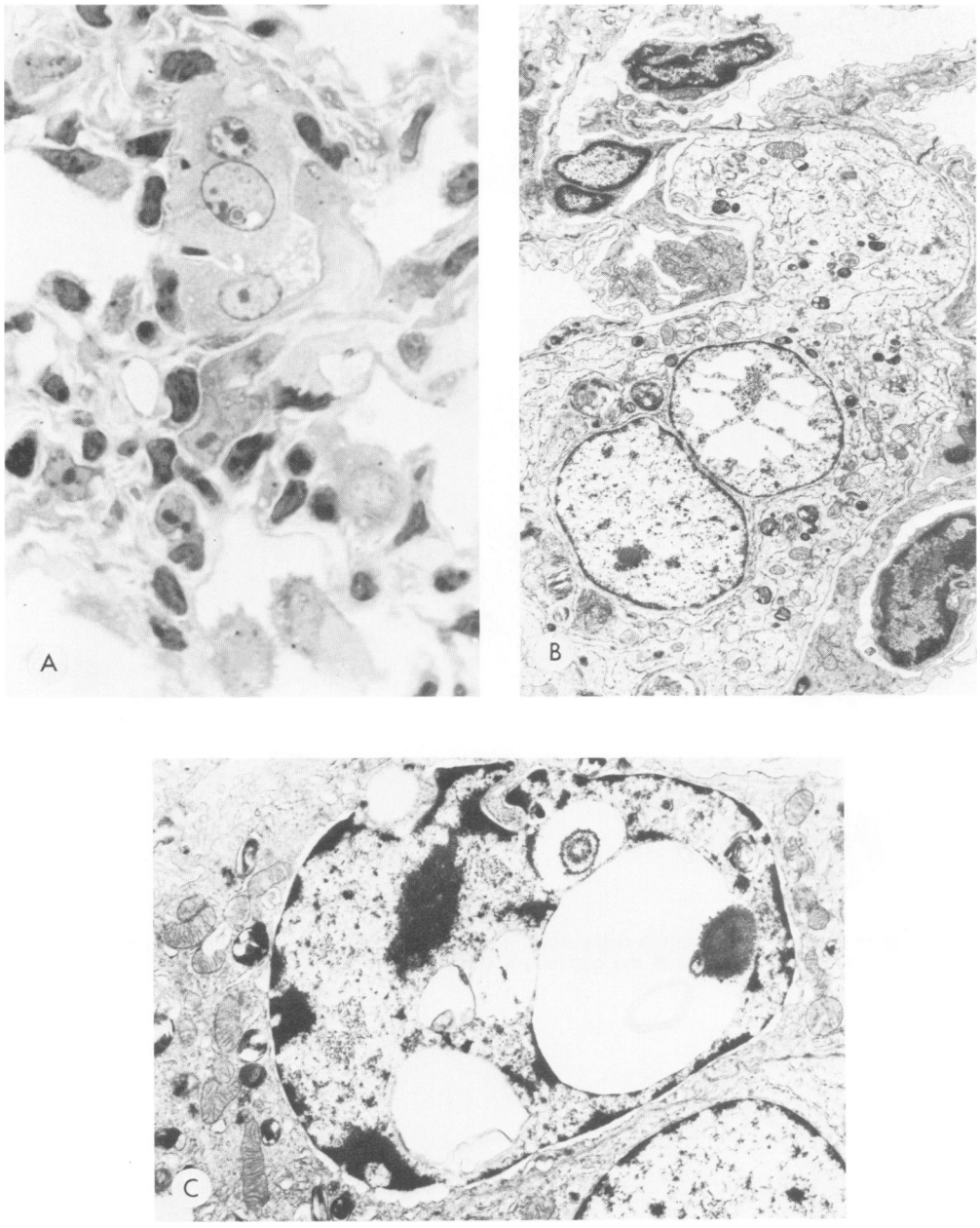
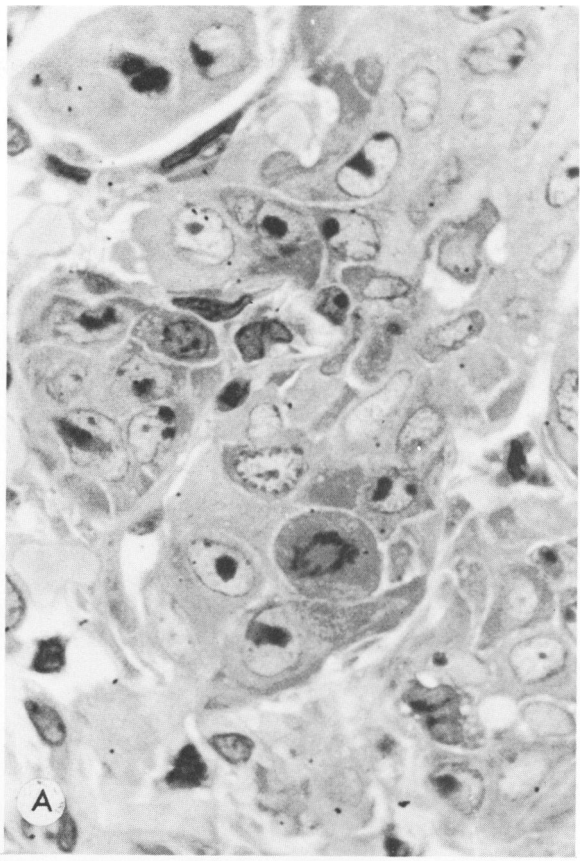


Figure 5—Eight weeks after multiple bleomycin given intraperitoneally. Some alveoli lined by large Type 2 epithelial cells, often binucleate with some nuclear disruption. **A**—Light microscopy. (×960) **B**—Electron microscopy. (×6000) **C**—Type 2 cell nucleus. (×10,000) (With a photographic reduction of 18%)



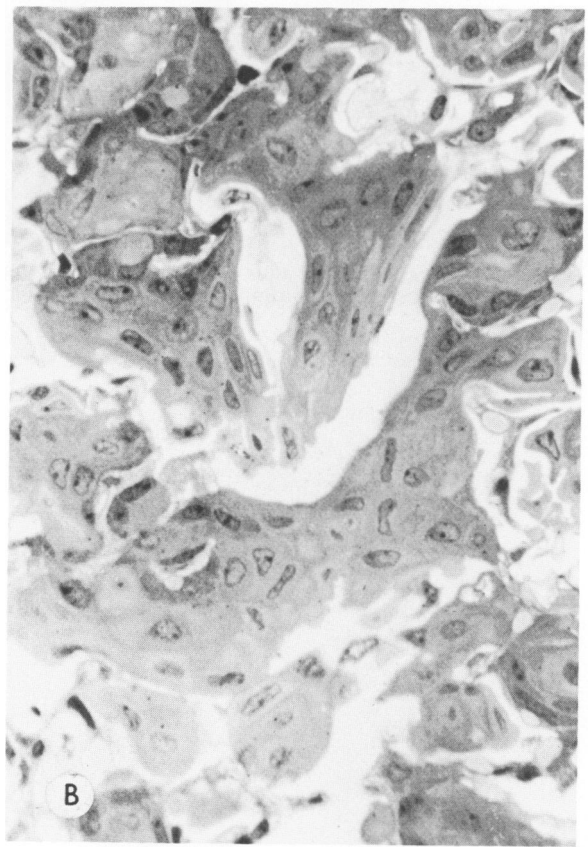


Figure 6—Twelve weeks after multiple intraperitoneal bleomycin. **A**—Tubules of epithelial cells line the alveoli; nucleoli are dense and a mitotic figure is seen. (×1100) **B**—Among the tubules, an area of squamous metaplasia is shown. (×960) (With a photographic reduction of 3%)

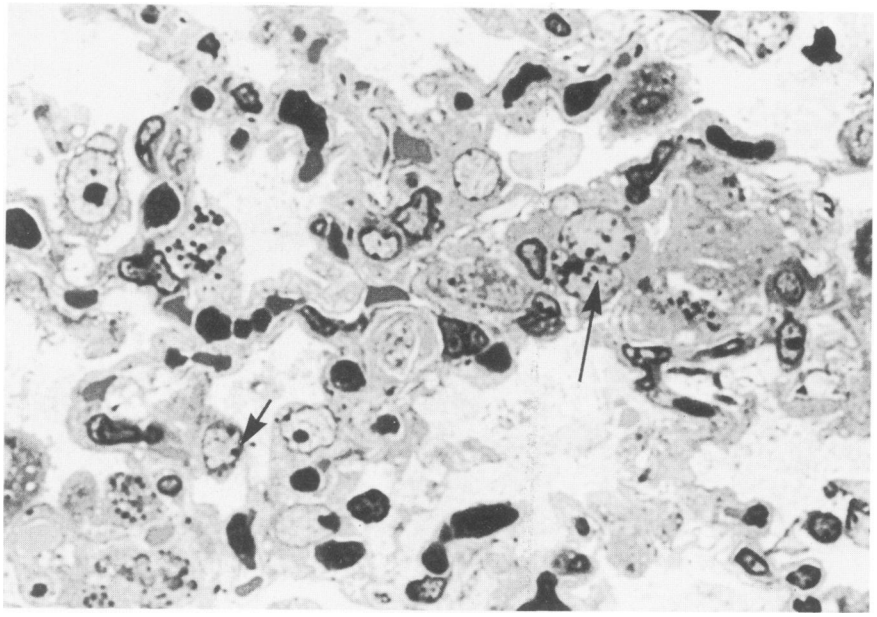


Figure 7—Autoradiograph after 8 weeks multiple intraperitoneal ^{3}H bleomycin. A few giant epithelial cells are shown, some with silver grains over the nuclei (*arrows*). (×960)