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LOUIS M.T. LIN

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PULMONARY LESIONS FOLLOWING ADMINISTRATION OF BUSULFAN TO MICE

APPROVED BY

Jacqueline J. Coalson

Walter Coalson

Richard Thomas Thrasher

Donald D. Holmes

John E. Allison

DISSERTATION COMMITTEE

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CHAPTER I

INTRODUCTION

Since the discovery of busulfan (myleran) in 1953 (Haddow and Timmis), this antineoplastic alkylating agent has been widely used in the treatment of chronic myelocytic leukemia. The main effectiveness and side effects of this drug have been well established from animal experiments and numerous clinical reports.

Busulfan is considered by investigators to induce no serious acute toxicity, and is thought to exhibit only delayed toxic effects, such as thrombocytopenia (Galton, 1953; Haut, et al., 1961), bone marrow depression (Unugur, et al., 1957; Galton, et al., 1958), hyperpigmentation (Galton, 1953; Calero, 1964), Addison-like syndrome (Kyle, et al., 1961; Harrold, 1966), gonadal atrophy (Galton and Till, 1955; Turesson, 1957; Galton, et al., 1958), pulmonary fibrosis (Oliner, et al., 1961; Leake, et al., 1963) and diffuse cytologic dysplasia (Gureli, et al., 1963; Ward, et al., 1965).

Pulmonary fibrosis associated with atypical epithelial

hyperplasia of the respiratory tract has been observed frequently by investigators, and is one of the most serious complications of busulfan therapy.

Despite the failure of Shimkin's early attempt to induce pulmonary tumors in mice (1954), the more recent literature describes and emphasizes an association between busulfan treatment and carcinoma of lung, breast, cervix, labium and vulva (Min and Gyorkey, 1968; Nelson and Andrews, 1964; Koss, 1965, 1968; Feingold and Koss, 1969). The question of a probable carcinogenic action of busulfan, therefore, must be considered.

The main objective of this animal study was to investigate the sequential long-term effects of busulfan on the lungs of mice.

CHAPTER II

LITERATURE REVIEW

Busulfan (myleran, 1,4-dimethanesulfonoxybutane)

Busulfan ($CH_3 \cdot SO_2 \cdot O \cdot CH_2CH_2CH_2CH_2 \cdot OSO_2 \cdot CH_3$) is a member of a group of compounds known as sulfonic acid esters. Its synthesis was the result of the research conducted by Haddow and Timmis (1953) in an attempt to enhance the therapeutic efficiency of nitrogen mustards and other alkylating agents. These workers assumed that the biological action of busulfan might resemble that of nitrogen mustards because of a common ability to alkylate. Experimentally, busulfan showed an intense inhibitory effect on the growth of Walker rat carcinoma 256 and a depressive influence on the myeloid series of both rat and man (Haddow and Timmis, 1953). Busulfan, unlike X-ray and nitrogen mustards, did not appreciably depress lymphocyte formation at a dose which caused a 50% or more decrease in the number of neutrophils (Elson, 1952). Haddow and Timmis (1953) thereby proposed that the relatively selective action of busulfan in depressing neutrophil production would be a desirable feature in clinical use for the control of myeloid leukemia.

Busulfan Therapy : Anti-leukemic Effects

Busulfan was introduced by Galton (1953) and then by Ledlie (1953), Wilkinson (1953), Bollag (1953) and Turesson (1953) into clinical use for the treatment of chronic myelocytic leukemia. The results of a two year trial of busulfan therapy in Galton's patients were summarized as symptomatic relief, splenic regression, reduction of leukocyte count and increase in hemoglobin level.

Because patients with chronic myeloid leukemia showed both clinical and hematological improvements with busulfan it was recommended as the drug of choice. During the succeeding years, busulfan was widely used clinically by many investigators in England (Hansen, 1954; Galton and Till, 1955; Blackburn, et al., 1956; Elson, 1958; Galton, et al., 1958), in the United States (Petrakis, et al., 1954; Levin, et al., 1954; Louis, et al., 1954; Haut, et al., 1955; Frelick, 1956; Frost and Jackson, 1956; Unugur, et al., 1957; Bethell, 1958; Dameshek, et al., 1958; Rundles, et al., 1959), in Japan (Moloney and Fujii, 1954), and in Australia (Kurrle, 1955). These workers obtained results comparable to those reported by Galton (1953), and confirmed that busulfan was of value in the treatment of chronic myelocytic leukemia. They concluded that busulfan treatment was superior to traditional radiotherapy and nitrogen mustard because of the simplicity of administration, absence of undesirable side effects, less erratic response, longer remissions, and occasional effec-

tiveness after other methods offered no further palliative therapy.

Busulfan also has been employed in other types of leukemia (Galton, 1953; Wilkinson, 1953; Petrakis, et al., 1956; Wintrobe, et al., 1954; Early and Prichard, 1956; Galton, et al., 1958; Rundles, et al., 1959), but was found ineffective. Even those patients with acute myelocytic leukemia and those with chronic myelocytic leukemia in acute myeloblastic relapse were usually not benefited by busulfan therapy (Galton, 1953; Ledlie, 1953; Bollag, 1953; Videback, 1955; Early, et al., 1956; Turesson, 1957; Galton, et al., 1958). From these numerous clinical experiences, busulfan has become a well-established form of palliative therapy for chronic myelocytic leukemia since 1960, and is used extensively worldwide.

Busulfan Therapy : Pulmonary Side Effects

Cytologic Abnormalities

Cytologic dysplasia and anaplasia in bronchial, bronchiolar, and alveolar lining cells was noted by several workers in leukemic patients subjected to prolonged busulfan therapy (Weston, et al., 1955; Waller, 1960; Gureli, et al., 1963; Nelson and Andrews, 1964; Ward, et al., 1965; Koss, et al., 1965; Feingold and Koss, 1969; Koss, 1968, Kirschner and Esterly, 1971). These authors believed that the cytotoxic effect of busulfan was responsible for widely distrib-

uted cytologic abnormalities.

The cytologic atypia is characterized by bizarre, large nuclei with coarse clumps of chromatin and prominent nucleoli. Sometimes multilobulated nuclei and multiple nucleoli were observed. It seemed significant that mitotic figures were not seen at the sites of nuclear abnormalities (Gureli, et al., 1963; Nelson and Andrews, 1964; Ward, et al., 1965), but the cytologic alterations did not differ in any visible way from nuclear pleomorphism and clumped chromatin on which cytologists use to diagnose dysplasia or malignancy (Ward, et al., 1965). The cellular and nuclear abnormalities in the dysplastic cells might well indicate the very early stages in the genesis of busulfan-induced cancer (Koss, et al., 1965; Feingold and Koss, 1969). This relationship was further supported by the fact that diffuse cellular atypia occasionally found in chronic myelocytic leukemia following busulfan therapy had been reported associated with other neoplasms (Angus and Gunz, 1963; Nelson and Andrews, 1964; Koss, et al., 1965; Koss, 1968; Min and Gyorkey, 1968; Feingold and Koss, 1969).

Interstitial and Intra-alveolar Fibrosis

Interstitial pulmonary fibrosis in patients with chronic myelocytic leukemia following busulfan therapy was first observed by Oliner, et al., (1961). These authors noted that pulmonary fibrosis with alveolar-capillary block might be seen following extensive radiation of the lungs

(Warren and Gates, 1940; Rodman, et al., 1960; Jennings and Arden, 1962; Spencer, 1968), but adrenal steroid therapy in the treatment of radiation fibrosis of lung invariably failed to reserve the course of the pulmonary lesions, although symptomatic relief might occur (Rubin, et al., 1958). In contrast, their patients showed striking resolution of symptoms and signs of pulmonary fibrosis after treatment with prednisone. Therefore, the development of pulmonary fibrosis during busulfan therapy was thought to be a hypersensitivity drug reaction (Oliner, et al., 1961). Diffuse interstitial pulmonary fibrosis associated with busulfan therapy in chronic myelocytic leukemia was afterwards described (Kyle, et al., 1961; Leake, et al., 1963; Harrold, 1966; Smalley and Wall, 1966; Boyles, 1969; Littler, et al., 1969). Leake and coworkers (1963) were also convinced that a hypersensitivity reaction to drug was the probable mechanism of pulmonary fibrosis.

A different opinion about the formation of pulmonary fibrosis was elaborated by Heard, et al., (1966) and Heard and Cooke (1968). They believed that pulmonary fibrosis following treatment with busulfan was largely intra-alveolar rather than interstitial, as had previously been described. Heard and Cooke (1968) observed that organizing fibrinous edema was common in patients after busulfan therapy but not in the other patients (Heard, et al., 1966). The earliest histological changes in lungs consisted of an intra-alveolar

accumulation of fibrin mixed with varying numbers of red cells, sometimes the fibrin was becoming organized by fibroblasts and replaced by reticulin and collagen.

Besides fibrinous edema and fibrosis, busulfan also induced the formation of large atypical alveolar lining cells which were found in patients receiving busulfan and in none of the patients not given busulfan (Heard and Cooke, 1968; Heard, et al., 1966). These investigators presumed that increased permeability of alveolar capillaries or venules was the common cause of fibrinous edema and intra-alveolar fibrosis, due to busulfan acting on the alveolar wall, either through the development of a hypersensitivity reaction or to an increased intravascular pressure.

Koss, et al., (1965) and Burns, et al., (1970, 1971) reported that the development of pulmonary fibrosis with marked cellular atypia of lining epithelial cells in chronic myelocytic leukemic patients following busulfan therapy was both interstitial and intra-alveolar. They speculated that the radiomimetic property of busulfan was the probable mechanism of producing pulmonary fibrosis.

Pulmonary fibrosis, generalized nuclear abnormalities and carcinoma of vulva were observed by Feingold and Koss (1969) in a case of chronic myelocytic leukemia after long-term administration of busulfan. These workers believed that pulmonary fibrosis and other findings pointed to radiomimetic effect of busulfan, since there was no histologic

evidence that the reaction was the result of hypersensitivity.

Min and Gyorkey (1968) described a case of bronchiolar cell carcinoma in a lung with diffuse interstitial pulmonary fibrosis and atypical alveolar cell hyperplasia following 5 years of busulfan therapy for chronic granulocytic leukemia in a 72-year-old black male. They stated that the giant nuclei in the dysplastic epithelial cells were characteristic of neoplastic changes, and were similar to those seen in the malignant cells of bronchiolar cell carcinoma. Therefore, Min and Gyorkey (1968) suggested that dysplasia and carcinoma were different stages of the same process, and dysplasia might precede the malignant change.

The presence of diffuse interstitial pulmonary fibrosis in Min and Gyorkey's case (1968) was also noteworthy. Steroid therapy had been instituted in the patient for a possible hypersensitivity reaction to busulfan, but without response. Min and Gyorkey thereby proposed that the possible mechanism of pulmonary fibrosis following busulfan therapy was radiomimetic property of busulfan, because the pathologic changes in irradiation pneumonia (Warren and Spencer, 1940; Smith, 1963) were similar to those seen in their case. The atypical epithelial cell changes and bronchiolar cell carcinoma were attributed to mutagenic and carcinogenic effect of busulfan (Min and Gyorkey, 1968).

The question of carcinogenicity of busulfan was raised

by the experimental observation of Upton, et al., (1961) who found that, if myleran was given to mice before and after whole X-ray irradiation, there was an increase in the incidence of thymic lymphomas. Angus and Gunz (1963), Nelson and Andrews (1964), Koss, et al., (1965), and Feingold and Koss (1969) all attributed carcinoma found in their chronic myelocytic leukemic patients to busulfan's effect, because their patients had received a large amount of busulfan.

Busulfan Therapy : Other Side Effects

Other side effects reported following prolonged busulfan administration have included: thrombocytopenia and bone marrow depression (Galton, 1953; Wilkinson, 1953; Bierman, et al., 1954; Elson, 1955; Haut, et al., 1955; Spurr, et al., 1956; Blackburn, et al., 1956; Hyman and Gellhorn, 1956; Galton, 1956; Unugur, et al., 1957; Hayhoe and Koh, 1957; Galton, et al., 1958; Galton, 1969), hyperpigmentation and Addison-like syndrome (Galton, 1953; Petrakis, et al., 1954; Galton, 1956; Spurr, et al., 1956; Louis, et al., 1956; Galton, et al., 1958; Desai, 1959; Wilkinson and Turner, 1959; Ritz and Krim, 1959; Vahlquist and Vuille, 1960; Bridge, et al., 1961; Kyle, et al., 1961; Persson and Soderstrom, 1962; Leake, et al., 1963; Calero, 1964; Kyle and Dameshek, 1964; Sprunt and Rizza, 1966; Smalley, et al., 1966, Harrold, 1966), gonadal atrophy (Galton and Till, 1955; Greig, 1956; Louis, et al., 1956; Turesson, 1957; Galton, et al., 1958; Wilkinson and Turner, 1959; Belohorsky, et al., 1960; Smalley and Wall,

1966; Bollag, 1953, 1954; Jackson, et al., 1959; Heller and Jones, 1964), teratogenic effect (Diamond, et al., 1960; Murphy, et al., 1958), cataract (Soloman, et al., 1955; Light, et al., 1956; Kandori and Kurimoto, 1960), and jaundice (Underwood, 1970, 1971).

CHAPTER III

MATERIALS AND METHODS

Animal Preparation

One hundred and forty weanling female strain A mice weighing approximately 10-12 gm purchased from Jackson Laboratory, Bar Harbor, Maine, were equally divided into control and treated groups. They were housed in twenty-eight plastic cages, five mice each and supplied with mouse and rat diet and tap water. After two weeks, injections were started in the mice (7-8 weeks of age). The treatment of the groups is outlined as follows:

1. Control group ------ intraperitoneal injection of 0.1 CC of 0.9% sodium chloride (saline solution).
2. Treated group ------ intraperitoneal injection of 0.5 mg busulfan dissolved in 0.1 CC of 0.9% sodium chloride.

Busulfan (Alrich Chemical Co., Inc., Milwaukee, Wisconsin) is a drug of white fine crystals, and is only slightly soluble in saline solution. Before injection, busulfan was dispersed in saline solution, warmed with hot tap

water and agitated constantly until the crystals were dissolved. The solution was cooled slowly at room temperature.

The two groups were injected twice a week during the duration of the experiment. After the first week of injection, equal numbers of both control and treated mice were sacrificed at one-week intervals for the first twelve weeks, and then at two-week intervals thereafter.

Mice were killed by cervical vertebral dislocation. To assure good preservation of pulmonary architecture, the lungs were fixed intrathoracically by endotracheal inflation with 4% glutaraldehyde in 0.08 M cacodylate buffer at pH 7.2-7.4 as indicated below.

After cutting through the skin of neck, thyroid gland and infrahyoid muscles were separated, and the trachea was exposed. Small scissors were utilized to separate the trachea from the esophagus, and a thread was passed behind the trachea and tied loosely near the bifurcation. The trachea was then lifted slightly, and 2 CC of glutaraldehyde was injected into the lumen of the trachea. During injection, the trachea and needle were clipped tightly with forceps to prevent glutaraldehyde escape. Following the perfusion of 2 CC of glutaraldehyde into the lungs, the trachea was tied quickly. Ten minutes later the thoracic cavity was opened, the lungs removed, placed in 4% cold glutaraldehyde, stored in the refrigerator, and then each lobe of both lungs trimmed separately for light and electron microscopic studies.

Tissue Preparation

Light Microscopy

After overnight storage in 4% cold glutaraldehyde, the tissues were washed in sucrose buffer (pH 7.2-7.4) for 30 minutes. The specimens were dehydrated, cleared and embedded in paraffin (Paraplast, Fisher). Sections of 5-6 microns were stained with Harris's hematoxylin-eosin and May-Grunwald-Giemsa according to the Armed Forces Institute of Pathology Manual. The prepared slides were examined with an A/O (American Optical) Spencer research microscope.

Electron Microscopy

For electron microscopic study, tissues were fixed overnight in 4% buffered glutaraldehyde, washed for 30 minutes in sucrose buffer, and then minced into 1 mm blocks. The samples were postfixed in 1% osmium tetroxide buffered with veronal-acetate buffer (pH 7.2-7.4) for 1 hour, washed in cold osmium buffer (pH 7.2-7.4) for 30 minutes, and then dehydrated in ascending alcohol concentrations (70, 95, 100 per cent for 15 minutes with 3 changes).

After alcohol dehydration, samples were divided into two halves. One half was embedded in Epon 812 and the other half in Spurr embedding medium. The procedure of embedding tissues in Epon 812 consisted of:

1. Infiltration with propylene oxide for 30 minutes.
2. Infiltration with a mixture of prcpylene oxide and

epoxy resin (1:1) for 1 hour with constant agitation in an automatic shaker.

3. Infiltration with pure epoxy resin mixture for 1 hour in the automatic shaker.
4. Embeddment in epoxy resin mixture in gelatin capsule.
5. Polymerization in 60°C oven for 24 hours.

The procedure of embedding tissues in Spurr embedding media (Spurr, 1969) consisted of:

1. Infiltration with a mixture of absolute alcohol and Spurr (2/3:1/3) for 20 minutes with constant agitation.
2. Further infiltration with a 1:1 mixture of absolute alcohol and Spurr for 1 hour with constant agitation.
3. Infiltration with pure Spurr for 1 hour in the shaker.
4. Embeddment in Spurr in gelatin capsules.
5. Polymerization in 60°C oven for 12 hours.

After polymerization, the prepared Epon and Spurr blocks were trimmed. Ultrathin sections were cut with a Porter-Blum MT-1 microtome and picked up on copper mesh grids, stained with uranyl acetate and lead citrate, and examined with a RCA EMU-3G electron microscope.

Mycoplasma Cultivation

Electron microscopic study of some of the lungs revealed that mycoplasmas were present, therefore 20 additional mice were used for the isolation of the mycoplasma. As be-

fore, intraperitoneal injection of busulfan (0.5 mg dissolved in 0.1 CC saline solution) was given to these mice twice a week. After 14 weeks of injection, mice were sacrificed at two-week intervals, 5 mice each time. Samples for mycoplasma isolation and immunofluorescence studies were obtained immediately at necropsy.

Media Preparation for Mycoplasma Isolation

Mycoplasma Agar (Hayflick, 1965):

A. Distilled water	700 ml
B. Difco Bacto-PPLO agar, dehydrated	35 gm
C. Yeast extract, 25%	100 ml
D. GG free horse serum (Gibco)	200 ml
E. Penicillin-G (Lilly)	1000 units/ml
F. Thallium acetate	1 mg/ml

The dehydrated agar was dissolved in distilled water, and the yeast added. The solution was then adjusted to pH 7.8 with 2% NaOH or HCl before autoclaving. Subsequently, horse serum, penicillin and thallium acetate were added to the solution and then poured into 50 x 20 mm sterilized Falcon plastic petri dishes with tight lids. Mycoplasma agar plates were dried in sterile air for 2 hours, inverted, and stored at 2°-8°C.

Mycoplasma Broth (Hayflick, 1965):

A. Distilled water	700 ml
B. Difco PPLO broth with CV, dehydrated	35 gm
C. Yeast extract, 25%	100 ml

D. GG free horse serum (Gibco) 200 ml

E. Penicillin-G (Lilly) 1000 units/ml

F. Thallium acetate 1 mg/ml

The procedure of preparing mycoplasma broth was similar to that of mycoplasma agar. Using sterile techniques, the broth was pipetted into sterilized test tubes with screw caps, 5 ml per tube and stored at 2° to 8°C.

Yeast Extract

A. Distilled water 1000 ml

B. Fleischmann's pure dry yeast, type 2040 250 gm

250 gm of dry yeast was added to 1000 ml of distilled water and heated to boiling. The solution was cooled and centrifuged at 10,000 RPM (revolutions per minute) for 30 minutes. The supernatant was removed and adjusted to pH 8 with 2% NaOH. It was then autoclaved, cooled, and re-centrifuged for 30 minutes at 10,000 RPM. The supernatant was placed in 50-100 ml tubes and frozen at -20°C until needed.

Isolation Technique

1. At necropsy, each mouse trachea was flushed with 2.0 ml mycoplasma broth, and 0.7 ml of the trachea wash was collected to inoculate the agar (0.4 ml) and broth (0.3 ml) cultures.

2. For each group (5 mice), one uninoculated broth tube and one agar plate were used for controls.

3. Both inoculated and uninoculated broth tubes and

agar plates were incubated at 37°C.

4. Agar plates were examined microscopically for appearance of growth, beginning with the first day of incubation through at least two weeks.

5. Broth cultures were checked everyday for a minimum of 5 days.

Immunofluorescence

Preparation of Frozen Sections

Lung tissues were placed in a large sterile test tube and quickly frozen in liquid nitrogen and stored at -20°C.

An Ames Lab-Tek cryostat was used to obtain the frozen sections. Frozen sections (6-8 microns) were cut and transferred to slides with a soft brush. The mounted sections were stored at -20°C until use.

Fixation

Sections were allowed to thaw and dry slightly, fixed for 10-30 minutes in cold acetone (4°C), washed thoroughly with PBS (phosphate buffered saline) pH 7.2 to 7.5, rinsed with distilled water and stained.

Staining and Mounting

A few drops of appropriately diluted hyperimmune rabbit antiserum to Mycoplasma pulmonis (Microbiological Associates, Bethesda, Maryland) were added to the tissue sections. The slides were then incubated in a wet chamber maintained

at room temperature for 30 minutes. Following incubation, slides were washed in PBS for 15 minutes with 3 changes, rinsed in distilled water and dried. Subsequently, slides were treated with diluted fluoresceinated goat anti-rabbit globulin (Difco Laboratories, Detroit, Michigan) and incubated for 30 minutes in a wet chamber at room temperature, washed in PBS, rinsed in distilled water and mounted in Difco FA mounting fluid, and analyzed using a Zeiss photomicroscope fitted with a dark-field condenser, mercury arc lamp (HBO 2,000 W), a Schott BG 12/min excitor filter, a UG 1 heat absorption filter and a 440-650 barrier filter.

Controls consisted of: (1) omitting the hyperimmune serum in the staining sequence and using the fluoresceinated globulin alone, (2) examination of untreated specimens to exclude autofluorescence.

CHAPTER IV

RESULTS

Light Microscopic Observations

In comparing the pulmonary findings between treated and control groups, it was found that busulfan-treated mice had marked pulmonary changes. This occurred in animals after 14 weeks of busulfan injection. Before 14 weeks, 2 of 25 busulfan-treated mice and 4 of 25 control mice showed mild, focal pulmonary changes. From 14 to 30 weeks, 26 of 45 treated mice were found to have diffuse, severe pulmonary lesions, and only 3 of 45 controls had localized lung changes (Table 1).

The earliest changes (1-13 weeks) in lungs of both groups was bronchial lymphoid hyperplasia, with subsequent bronchial accumulation of purulent exudate, alveolar influx of macrophages and polymorphonuclear leukocytes, and epithelial hyperplasia. Fibrinous edema was noted at this stage in the treated mice but not in controls. Interstitial fibrosis and epithelial dysplasia, seen in treated group, occurred during the 14 to 30 week time period.

In 28 of the 70 busulfan-treated mice exhibiting any type of pulmonary change, 11 showed interstitial fibrosis, 7

TABLE 1

COMPARISON OF PULMONARY LESIONS IN CONTROL AND BUSULFAN-TREATED MICE

Time after treatment (week)	No. of mice sacrificed	No. of mice with pulmonary lesions	
		Control group	Treated group
2	4	0	2
4	4	1	0
6	4	2	0
8	4	0	0
10	5	0	0
12	5	1	0
14	5	1	4
16	5	0	4
18	5	1	5
20	5	0	2
22	5	0	2
24	5	0	1
26	5	0	3
28	5	0	3
30	5	1	2
	Total 70	7	28

alveolar cell dysplasia, 4 bronchial cell dysplasia and 4 pulmonary adenomas. None of the above four changes was found in controls. The type of pulmonary changes observed in both control and treated animals is summarized in Table 2.

The distinct changes in the lungs of busulfan-treated mice are described in detail in the following pages.

Alveolar Spaces

The alveolar spaces were filled with either inflammatory cells consisting of polymorphonuclear leukocytes, macrophages and mononuclear cells, or edema fluid mixed with fibrin (Figure 1). When fibrinous edema had become organized and incorporated into alveolar walls, the alveoli were completely obscured by proliferation of fibroblasts and fibrous connective tissue (Fig. 2).

Outpouring of large, foamy macrophages was very conspicuous. They were scattered singly or in groups throughout the alveolar spaces (Fig. 3).

Alveolar Cells

The alveolar cells showed marked hyperplastic change, especially in the area of inflammation, and the hyperplastic cells formed a pseudo-stratified epithelium projecting into the alveolar space (Fig. 4). Because the cell boundary of these hyperplastic alveolar cells was not clearly defined, they resembled giant multinucleated cells.

In general, the hyperplastic alveolar cells were

TABLE 2

TYPE OF PULMONARY LESIONS OBSERVED IN EXPERIMENTAL MICE

Type of pulmonary lesions	Treated group (70 mice)	Control group (70 mice)
Peribronchial lymphoid hyperplasia	20	4
Bronchial accumulation of purulent exudate	16	2
Alveolar influx of macrophages and polymorphonuclear leukocytes	20	5
Fibrinous edema	6	0
Bronchial hyperplasia	11	2
Alveolar cell hyperplasia	15	4
Interstitial fibrosis	11	0
Bronchial dysplasia	4	0
Alveolar cell dysplasia	7	0
Pulmonary adenoma	4	0

larger than usual, having abundant cytoplasm with single or multiple nuclei. The cell as a whole was not markedly distorted; however, in some areas the epithelial proliferation was so abnormal that cytologic atypia was observed (Fig. 5). The cell nuclei were very large with irregularly clumped chromatin and sometimes exhibited prominent nucleoli. Mitoses were present in these abnormally hyperplastic cells (Fig. 6).

Metaplasia to squamoid cells (Fig. 7) or to low ciliated columnar epithelial cells was frequently seen in alveolar lining epithelium.

Four pulmonary tumors were found in mice receiving busulfan for 17, 25, and 27 weeks. Three of these tumors were located in a subpleural position and appeared to arise from alveoli devoid of inflammatory changes (Fig. 8). A fourth tumor was found associated with the primary bronchus (Fig. 10). The three peripheral tumors were sharply circumscribed and compressed the surrounding alveoli. The fourth tumor lacked a sharply defined border. However, all four tumors were identical cytologically. The cells were uniform in size and shape and possessed a deep eosinophilic cytoplasm with either uniform dark or vesicular nuclei, and formed papillary or glandular structures (Fig. 9). There was no evidence of malignancy.

Alveolar Walls

Most alveolar walls did not show remarkable thicken-

ing. However, in some cases alveolar septa were broadened due to one of the following: (1) infiltration of lymphocytes and plasma cells (Fig. 11), (2) proliferation of fibroblasts and fibrous connective tissue with a moderate degree of mononuclear cell infiltration (Fig. 13), and (3) deposition of fibrin, infiltration of polymorphonuclear leukocytes and mononuclear cells and interstitial edema (Fig. 14). Frequently, severe fibrous thickening of alveolar walls was found associated with cellular atypia of lining epithelial cells, especially those in obsolete alveoli (Fig. 15). The lining cells were swollen, giant and multinucleated, and had large hyperchromatic nuclei.

Mononuclear cell infiltration of alveolar walls was also occasionally noted in control mice (4 of 70) (Fig. 12).

Bronchi and Bronchioles

The bronchi and bronchioles were surrounded by lymphocytes and plasma cells. In severely affected air passages, the lumen of bronchi was markedly dilated and completely or partly plugged with pus cells and necrotic cellular debris (bronchiectasis) (Fig. 16).

When inflammation of bronchi and bronchioles occurred, hyperplasia of respiratory epithelium was very remarkable. The bronchial epithelium was either proliferative to true stratification, or in some instances metaplastic to squamous cells simulating the appearance of carcinoma <u>in situ</u> (Fig. 17), but mitotic figures were not present. In bronchioles,

the epithelial growth occasionally invaded adjacent alveoli through the basement membrane and muscularis mucosae (Fig. 18).

Blood Vessels and Lymphatics

When mononuclear cell infiltration was limited to bronchi and bronchioles, only adjacent vessels were involved. As the inflammatory process advanced into lung parenchyma, all vascular channels showed prominent perivascular cuffing of lymphocytes and plasma cells. Sometimes, inflammatory cell infiltration in the blood vessel walls was so intense that destruction of endothelium could be observed.

Perivascular edema was noted in both busulfan-treated and control mice at the early stage of pulmonary changes (1-13 weeks), but no vasculitis was demonstrated.

Enlarged perivascular lymphatics were seen around all inflammed vessels.

Electron Microscopic Observations

The most striking findings in electron microscopy were the massive numbers of mycoplasma organisms inside the respiratory tract and the severe destruction of alveolar tissue in busulfan-treated mice. Mycoplasmas were present on the luminal surface of damaged bronchi, and were either closely associated with plasma membrane or cilia and microvilli of the lining epithelial cells (Fig. 19). Organisms were also observed within the phagocytic vacuoles inside the cytoplasm

of polymorphonuclear leukocytes and macrophages (Fig. 20).

The alveoli were filled with polymorphonuclear leukocytes, macrophages and desquamated type II pneumocytes (Fig. 22). Numerous crystal-like spicules, either free or contained in phagocytic vacuoles, were noted in the cytoplasm of alveolar macrophages from the lungs of busulfan-treated mice (Fig. 23).

Hyperplasia of alveolar type II cells with characteristic osmiophilic lamellar bodies was very prominent in the alveolar lining epithelium (Fig. 24). Collagen fibrils were found to be markedly increased in the alveolar walls of busulfan-treated mice (Fig. 25).

In both treated and control mice (1-13 weeks), the cytoplasm of endothelial cells was swollen and vacuolated. These changes were more marked in treated mice than in controls after 14 weeks of busulfan treatment.

Mycoplasma was not seen in the respiratory tract of control mice (Fig. 21).

Mycoplasma Cultivation

Mycoplasma pulmonis was cultured from the trachea of all mice (15) in group II, III, and IV which had received busulfan treatment for 16, 18, and 20 weeks respectively. M pulmonis was recovered from only 3 of the 5 mice in group I which had been treated for 14 weeks. The results of mycoplasma cultivation are summarized in Table 3.

TABLE 3

RESULTS OF MYCOPLASMA CULTIVATION AND IMMUNOFLUORESCENT STUDIES

No. of animals sacrificed	Weeks after busulfan injection	Mycoplasma pulmonis isolated from trachea	Positive immunofluorescent test
5	14	3/5*	5/5#
5	16	5/5	4/5
5	18	5/5	5/5
5	20	5/5	5/5

*Animals with Mycoplasma pulmonis/total studied.

#Animals with positive immunofluorescent test/total animals studied.

Immunofluorescence

Lungs from 19 of the 20 busulfan-treated mice used for mycoplasma isolation were immunofluorescent positive for *M pulmonis* (Table 3). The fluorescence appeared as a distinct zone of apple green color along the luminal surface of the hyperplastic bronchial and bronchiolar epithelium or as scattered granules in the luminal inflammatory exudate (Fig. 26).

CHAPTER V

DISCUSSION

The range of lesions observed in the lungs of control and treated mice was extensive and included peribronchial accumulations of lymphocytes and plasma cells, plugging of the bronchial lumina with purulent exudate, influx of polymorphonuclear leukocytes and alveolar macrophages into alveoli, and hyperplasia of respiratory epithelium. The changes indicated above are consistent with the histological lesions of CRD (chronic respiratory disease) in rats and mice (Nelson, 1957; Newberne, et al., 1961; Kohn and Kirk, 1969; Lindsey, et al., 1971). Interstitial and intra-alveolar fibrosis, dysplasia and anaplasia of bronchiolar and alveolar lining epithelia were seen only in the treated group.

Electron microscopy revealed numerous mycoplasma present in the respiratory tract with many organisms attached to the surface of bronchial epithelial cells. Cultivation and immunofluorescent studies demonstrated that the organisms were Mycoplasma pulmonis. Kohn and Kirk (1969) and Lindsey, et al., (1971) believed that this species was the primary pathogen responsible for CRD or rodent pulmonary mycoplasmo-

sis, and the findings in this study would support their work. However, in view of the marked difference in the development of lung lesions between busulfan-treated and control mice, it was unlikely that M pulmonis was the primary etiologic agent. Because both treated and control groups were housed in the same environment, they would be expected to exhibit an almost equal incidence of CRD, unless some antecedent damage to pulmonary structures and/or function had occurred. Based on the observation of higher incidence of pulmonary lesions in treated mice, the cytotoxic effect of busulfan was the only plausible etiologic factor to be considered.

Busulfan has been demonstrated to cause granulocytopenia (Galton, 1953; Galton, et al., 1958), atypical hyperplasia of bronchial and alveolar epithelium, and pulmonary fibrosis (Oliner, et al., 1961; Koss, et al., 1965; Min and Gyorkey, 1968). A decreased leukocyte count would deprive the individual of a basic defense mechanism and would render it more vulnerable and predisposed to infection. Abnormal hyperplasia of bronchial and alveolar epithelia would hinder the natural protective mechanism of respiratory tract and provide a favorable field for bacterial and viral growth.

In addition to the M pulmonis infection in the lungs of treated mice, the other pulmonary findings were: pulmonary fibrosis and associated atypical bronchiolar and alveolar cell hyperplasia. These two lesions have been noted in human patients undergoing busulfan treatment for chronic

myelocytic leukemia, and Heard and Cooke (1968) and Litter, et al., (1969) have used the term "busulfan lung" to characterize these changes.

Heard, et al., (1966) and Heard and Cooke (1968) noted that fibrinous edema in alveoli was a common occurrence after busulfan therapy. The persistent fibrinous edema subsequently underwent organization and incorporation into alveolar walls, so that the pulmonary fibrosis following busulfan therapy was largely intra-alveolar rather than interstitial. In this study, edema fluid mixed with fibrin was present in the alveoli of several busulfan-treated mice and in some areas the fibrinous edema had organized and had incorporated into alveolar walls as a proliferation of fibroblasts and mature connective tissue. However, in some mice marked fibrinous thickening of alveolar walls was seen without accompanying fibrinous edema and intra-alveolar fibrosis. This pattern is very similar to the interstitial pulmonary fibrosis described in chronic myelocytic leukemic patients following busulfan treatment (Oliner, et al., 1961; Leake, et al., 1963; Smalley and Wall, 1966; Boyles, 1969). Electron microscopy also confirmed that collagen fibers were markedly increased in the alveolar walls of busulfan-treated mice.

Oliner, et al., (1961), Heard, et al., (1966) and Heard and Cooke (1968) noted fibrinous edema with vasculitis in human patients receiving busulfan therapy and attributed this response to endothelial damage. Although a very mild

perivascular edema was noted in experimental animals, this change can not be attributed to busulfan therapy, since control animals also exhibited this change. Fibrinous edema, however, occurred only in busulfan-treated animals and only in areas showing severe inflammatory change. Ultrastructurally, endothelial morphologic criteria do not support the hypothesis of endothelial damage, and the appearance of fibrinous edema should be considered an indirect rather than a direct effect of busulfan on endothelial cells.

In association with pulmonary fibrosis, atypical epithelial hyperplasia was observed frequently in the lungs of busulfan-treated mice. The CRD complication in treated mice could have contributed to the epithelial hyperplasia seen in the respiratory tract (Newberne, et al., 1961). However, the interstitial and intra-alveolar fibrosis associated with large atypical alveolar lining cells and the markedly abnormal bronchiolar and alveolar cell hyperplasia are much more characteristic effects of busulfan (Koss, et al., 1965; Min and Gyorkey, 1968; Feingold and Koss, 1969; Burns, et al., 1970, 1971) than of mycoplasma.

Atypical epithelial hyperplasia was found in the cells lining bronchioles and alveoli. In the bronchioles, the epithelial cells sometimes extended through the basement membrane and muscularis mucosae into adjacent alveoli. This lesion was difficult to distinguish from true cancerous growth. Another pattern consisted of squamous epithelium

with dysplasia simulating carcinoma *in situ*. In the alveoli, the alveolar cell hyperplasia was diffuse, irregular and dysplastic. The individual epithelial cells were large, having giant nuclei with clumps of chromatin or prominent nucleoli but no histologic pattern conclusive of true neoplasia. The cytologic alterations in these hyperplastic epithelia were consistent with marked dysplastic changes. It is perhaps significant that mitotic figures were noted at the sites of cellular atypia since this finding has not been reported by previous investigators in the human busulfan lung (Gureli, *et al.*, 1963; Nelson and Andrews, 1964; Ward, *et al.*, 1965).

The incidence of pulmonary tumors induced by busulfan in this animal experiment is difficult to evaluate since strain A mice have been reported to carry a 40%-50% incidence of spontaneous lung tumors after age of 6 months. Although only 4 pulmonary adenomas (5.5%) were found in mice sacrificed after 17, 25, and 27 (2 tumors) weeks of busulfan treatment, the low incidence becomes more significant when compared with control animals. Pulmonary tumors were never found in control mice during this 30 week study. Unfortunately, the spontaneous pulmonary tumors in strain A mice and the drug-induced pulmonary adenoma are histologically indistinguishable (Stewart, 1966). At this time, it can not be stated with certainty whether the tumors are spontaneous or busulfan-induced.

Because of the finding of vasculitis in the human

busulfan lung, the probable mechanism of pulmonary fibrosis was thought by some investigators (Oliner, et al., 1961; Leake, et al., 1963; Heard and Cooke, 1968) to be due to the development of drug hypersensitivity. A second mechanism postulated for the propensity of busulfan to produce pulmonary fibrosis was its radiomimetic property which could induce a form of radiation fibrosis (Koss, et al., 1965; Min and Gyorkey, 1968; Feingold and Koss, 1969; Burns, et al., 1970, 1971). In this animal experiment, there was no evidence of a necrotizing vasculitis or fibrinoid connective tissue changes which have been described in drug associated hypersensitivity diseases. The data obtained from busulfan-treated mice are in agreement with the radiomimetic hypothesis since long standing edema elicits fibrotic change.

The cytologic atypia observed in bronchiolar and alveolar lining cells of treated mice can be attributed to arrest of cell mitosis. Busulfan is a bifunctional alkylating agent (Haddow and Timmis, 1953). In biological systems it has been shown to be capable of crosslinking or breaking two complementary strands of nuclear DNA, thus resulting in interferference with DNA replication and normal cell division (Lawley, 1957; Stacey, et al., 1958; Kohn, et al., 1966). Chromosomal fragmentation and suppression of the mitotic mechanism after busulfan administration have been observed by Koller (1958) and Alexander, et al., (1961). A tissue culture study by Chevremont, et al., (1960) of busulfan-treated fibroblasts

indicated that the metabolism of DNA was profoundly modified and that it stimulated an increase in nuclear Feulgen-positive material with polyploidy. They reported that the synthesis of DNA and protein was not disrupted in the busulfan-treated fibroblasts and that treated cells could not undergo complete cell division. This observation was verified for other alkylating agents by Powers and Pomeroy (1958). Under these conditions, the cells would become polyploid and more and more enlarged due to the inability to complete cell division. The results obtained in these two in vitro studies suggest that these mechanisms are involved in producing the cellular atypia observed in the lungs of busulfan-treated mice.

In this animal experiment, endothelial damage was expected in busulfan-treated mice with fibrinous edema and fibrosis. However, increased capillary permeability has been shown not always correlated by endothelial morphologic criteria. Because of the low incidence of pulmonary tumors in busulfan-treated mice, the carcinogenicity of busulfan is not conclusive but suspicious, for no lung tumor was found in controls. The experimental data suggests that busulfan acts directly or indirectly in causing pulmonary fibrosis through its radiomimetic effect. It should also be noted that the lungs of busulfan-treated mice were very vulnerable to mycoplasma infection.

CHAPTER VI

SUMMARY

The histologic changes observed in the lungs of mice, following busulfan administration, support the clinical findings that busulfan is capable of inducing pulmonary fibrosis, cytologic dysplasia of respiratory epithelium and probable lung tumors.

In 70 busulfan-treated mice examined, 40% showed microscopic lesions in the lungs, among them 9% had fibrinous edema, 16% had interstitial fibrosis, 6% had bronchiolar cell dysplasia, 10% had alveolar cell dysplasia and 6% had adenomas. None of these lesions was seen in 70 controls.

The mechanism of pulmonary fibrosis is postulated to be caused by increased permeability of alveolar capillaries probably through the action of busulfan's (indirect) radiomimetic property.

The cytologic dysplasia of bronchiolar and alveolar epithelium is due to the antimitotic effect of busulfan on epithelial cells.

It was noted in this experiment that the lungs of busulfan-treated mice were very susceptible to mycoplasma infection.

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APPENDIX

FIGURE LEGEND

AC II	type II alveolar cell (pneumocyte)
BM	basement membrane
Ci	cilia
CF	collagen fibrils
CS	crystal-like spicules
De	desmosome
Ep	epithelium
L	lysosome
LB	lamellar body
M	mitochondria
Mac	macrophage
MP	Mycoplasma pulmonis
Mv	microvilli
N	nucleus
Poly	polymorphonuclear leukocyte
PV	phagocytic vacuoles

PLATE I

Figure 1. The alveolar spaces are filled with polymorphonuclear leukocytes, macrophages, and edema fluid which contains a moderate amount of fibrin (14 weeks of busulfan administration. Hematoxylin and eosin. 220x).

Figure 2. The persistent fibrinous edema in the alveoli shows organization and incorporation into alveolar walls. The alveoli are completely obscured by proliferation of fibroblasts, connective tissue and infiltration of mononuclear cells (16 weeks of busulfan administration. H&E. 220x).

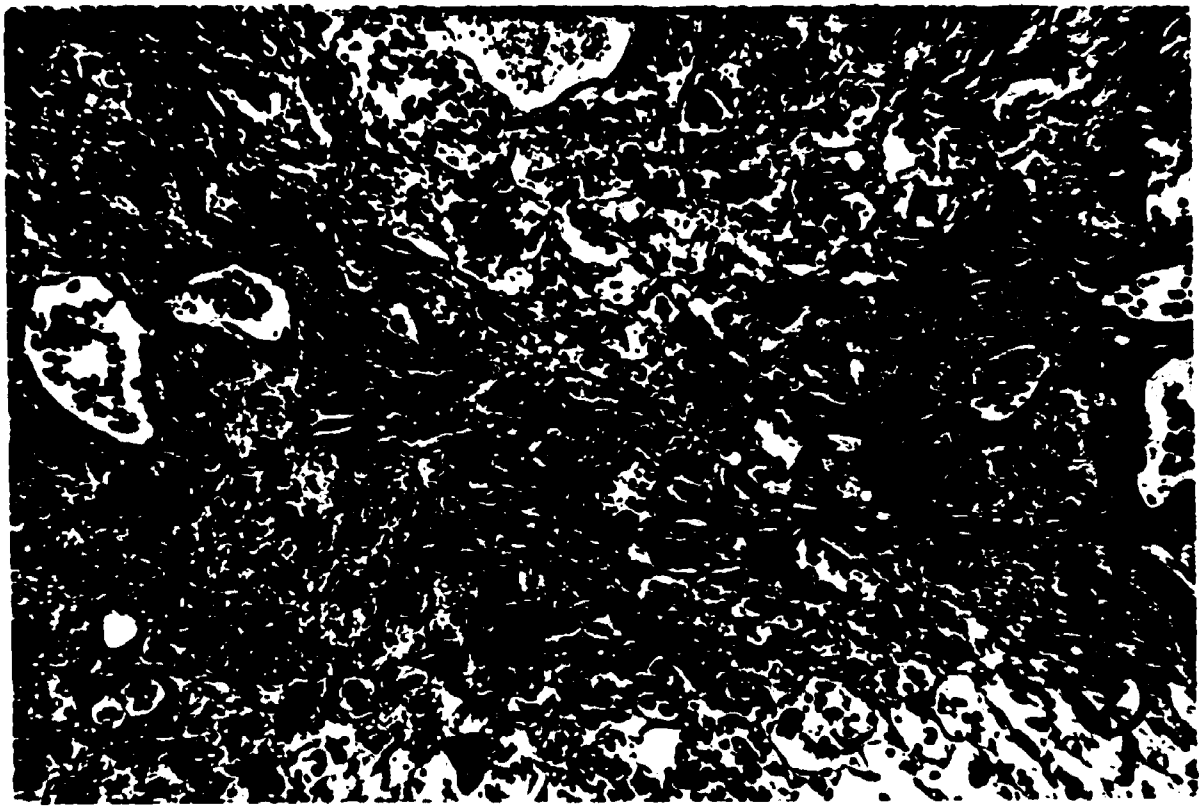
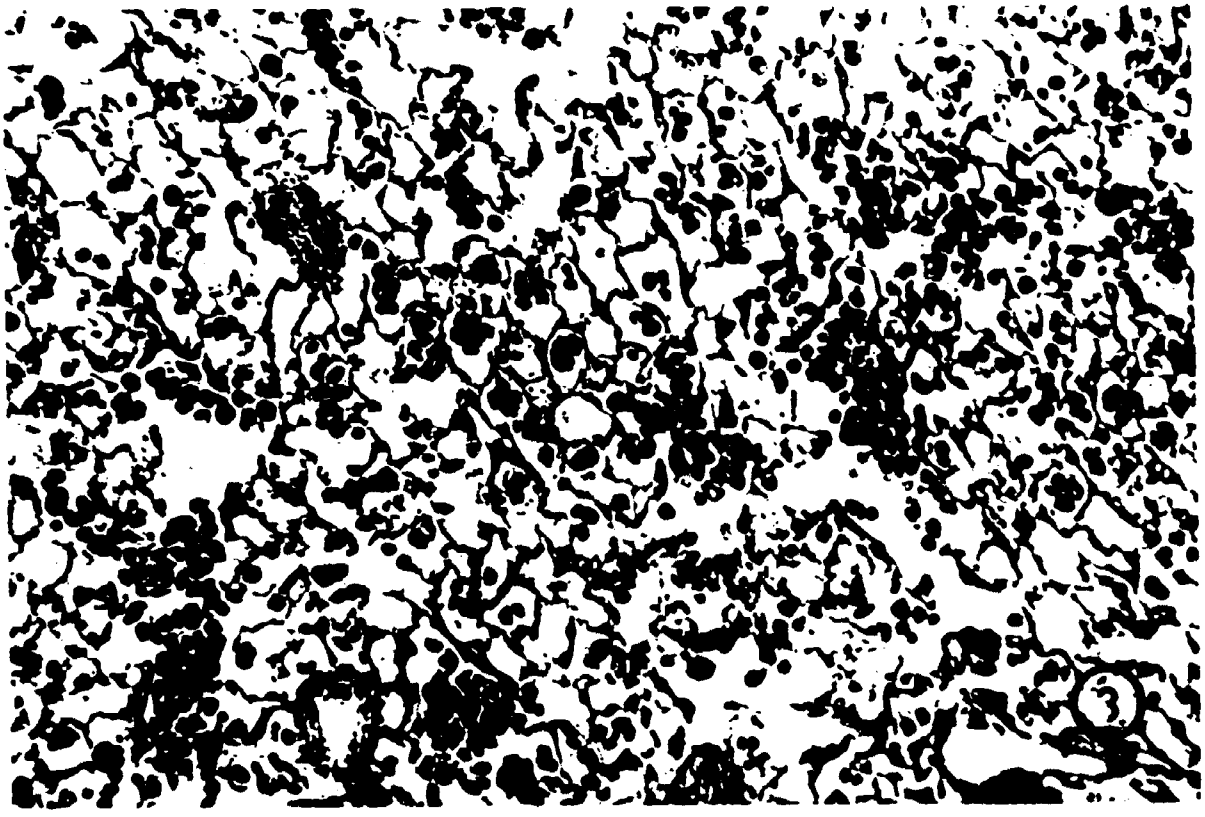


PLATE II

Figure 3. The section shows marked outpouring of large, foamy macrophages in the alveoli (30 weeks of busulfan administration. H&E. 220x).

Figure 4. Alveolar cell hyperplasia is very marked in areas of inflammation, and forms a pseudostratified epithelium that extends into alveoli. The cytoplasmic boundaries of the hyperplastic alveolar cells are poorly defined, so they appear as giant cells with multiple nuclei (16 weeks of busulfan administration. H&E. 220x).



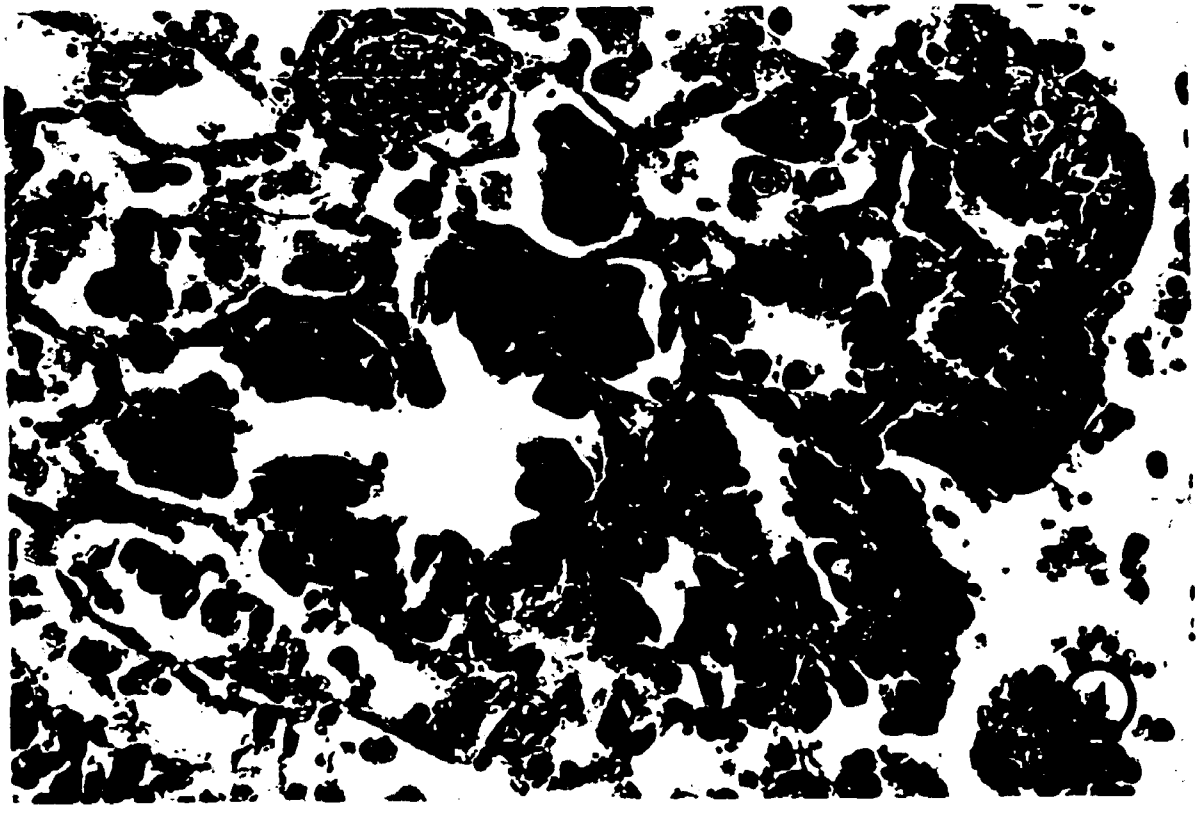
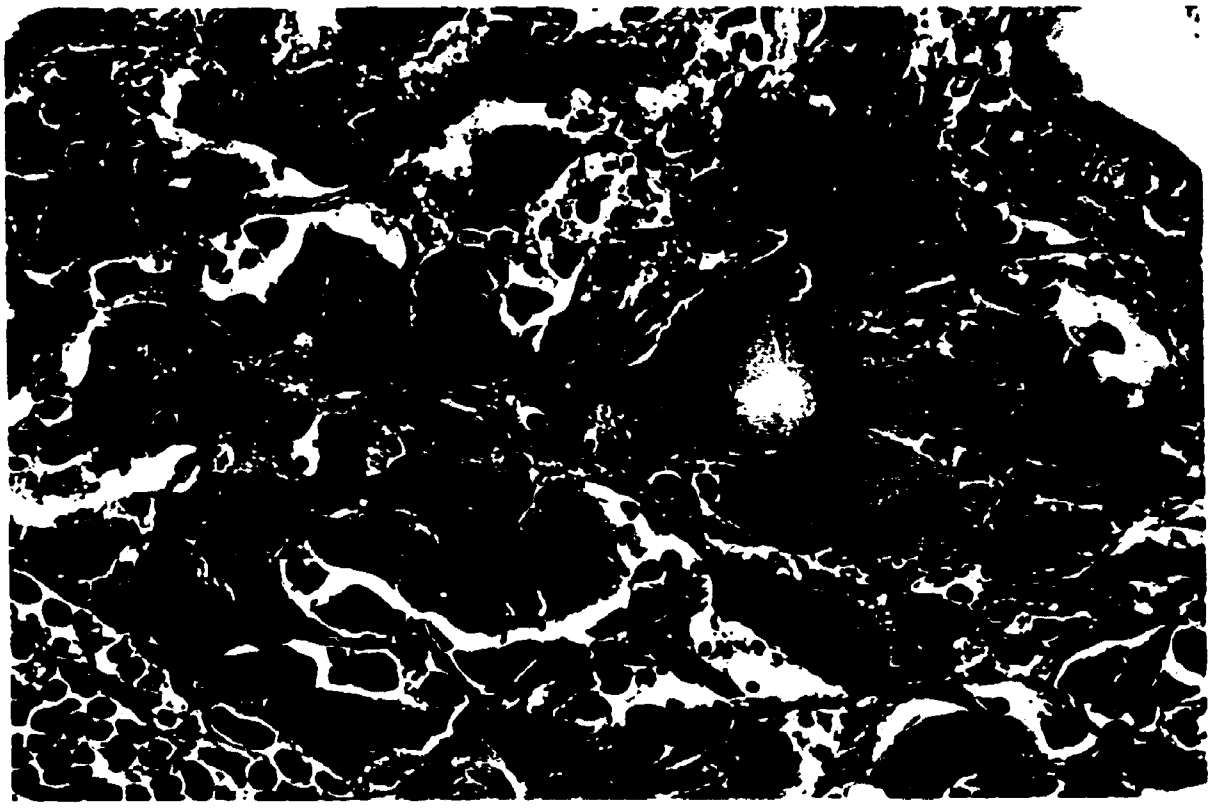


PLATE III

Figure 5. Atypical hyperplasia of alveolar cells is depicted. The nuclei of epithelial cells are very large with clumping of chromatin (15 weeks of busulfan administration. H&E. 500x).

Figure 6. This photomicrograph shows an area of diffuse and irregular proliferation of alveolar cells. Note the presence of mitotic activity in the field (15 weeks of busulfan administration. H&E. 500x).



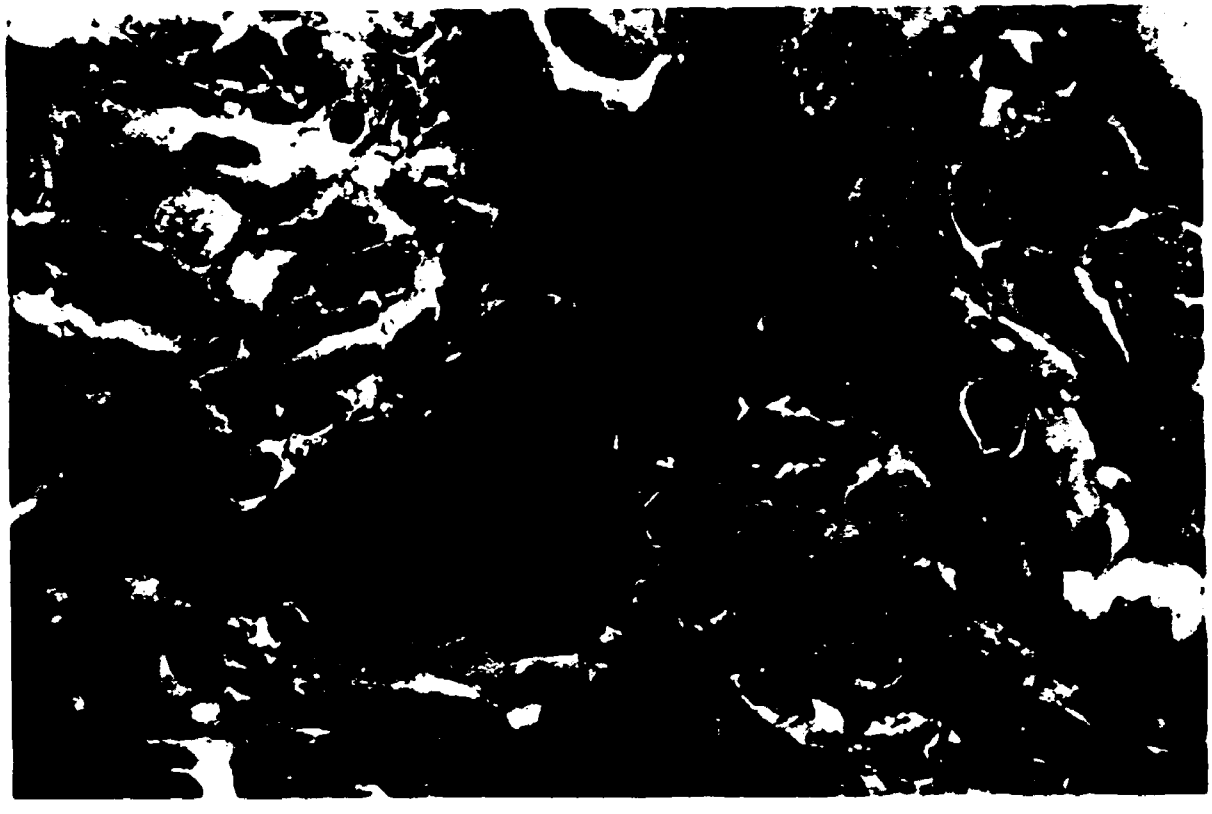
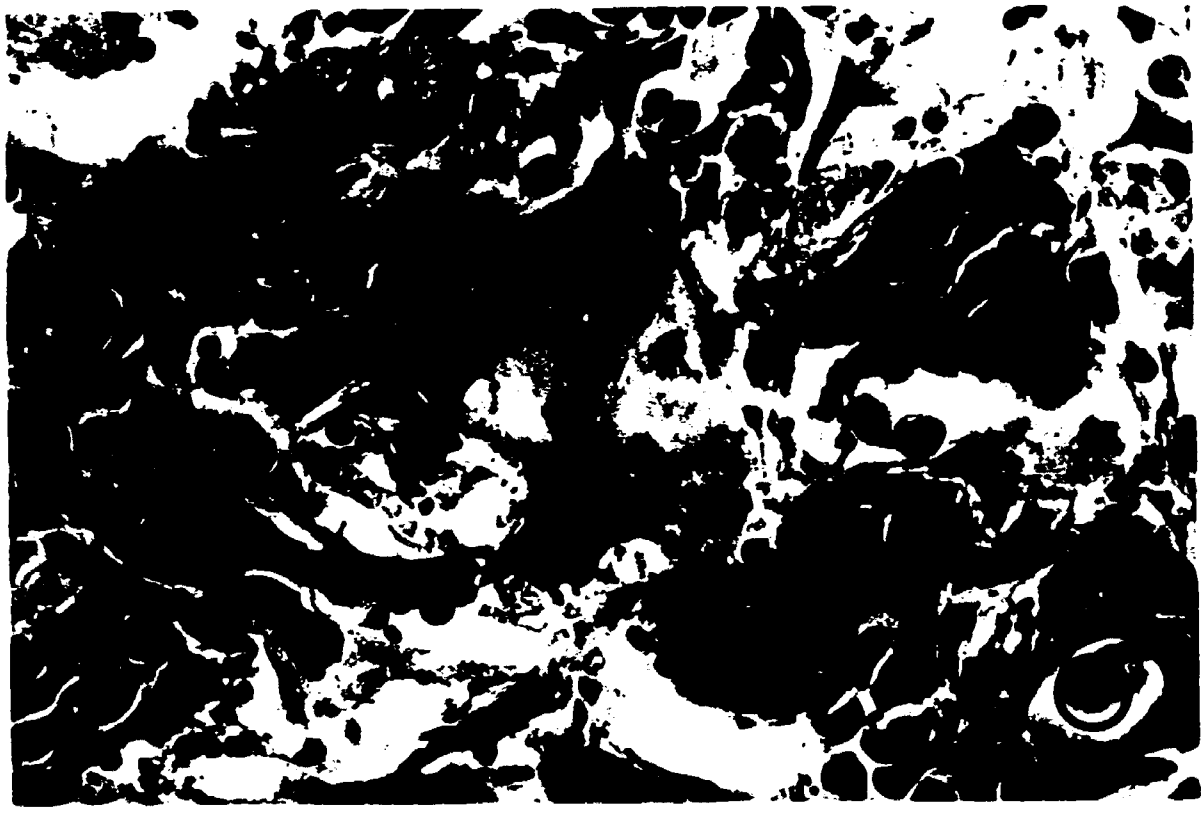


PLATE IV

Figure 7. Squamoid metaplasia of alveolar cells is depicted. There is marked variation in the size of hyperchromatic nuclei (15 weeks of busulfan administration. H&E. 500x).

Figure 8. A pulmonary adenoma situated beneath the pleura is seen. The tumor is well circumscribed and compresses the surrounding alveoli. The hyperplastic alveolar cells form pseudo-stratified epithelium with papillary projections (25 weeks of busulfan administration. H&E. 220x).



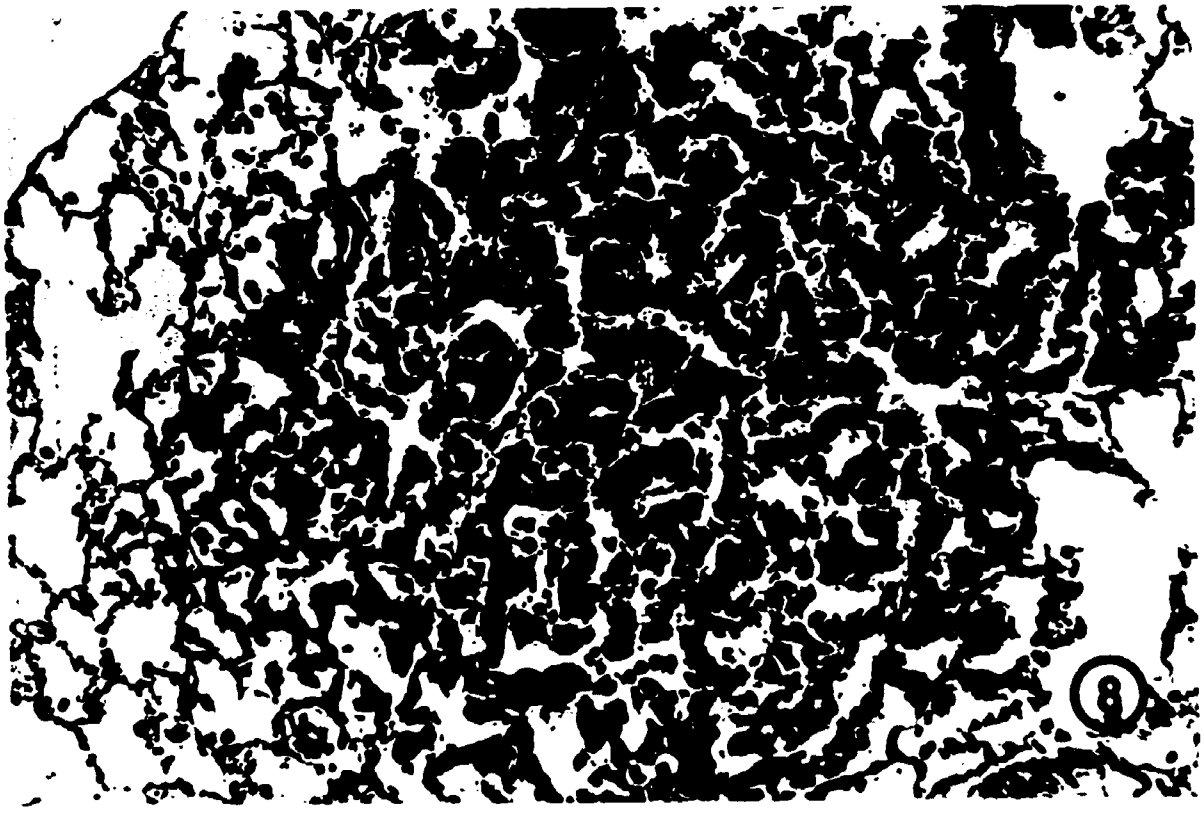


PLATE V

Figure 9. Higher magnification of Fig. 8 which shows the papillary formations of neoplastic alveolar cells. The cells are uniform in size and shape, having homogeneous, deeply eosinophilic cytoplasm with uniform dark nuclei (25 weeks of busulfan administration. H&E. 500x).

Figure 10. Demonstration of the close association of a pulmonary adenoma and a large bronchus is depicted. Note both papillary and glandular structures (17 weeks of busulfan administration. H&E. 220x).

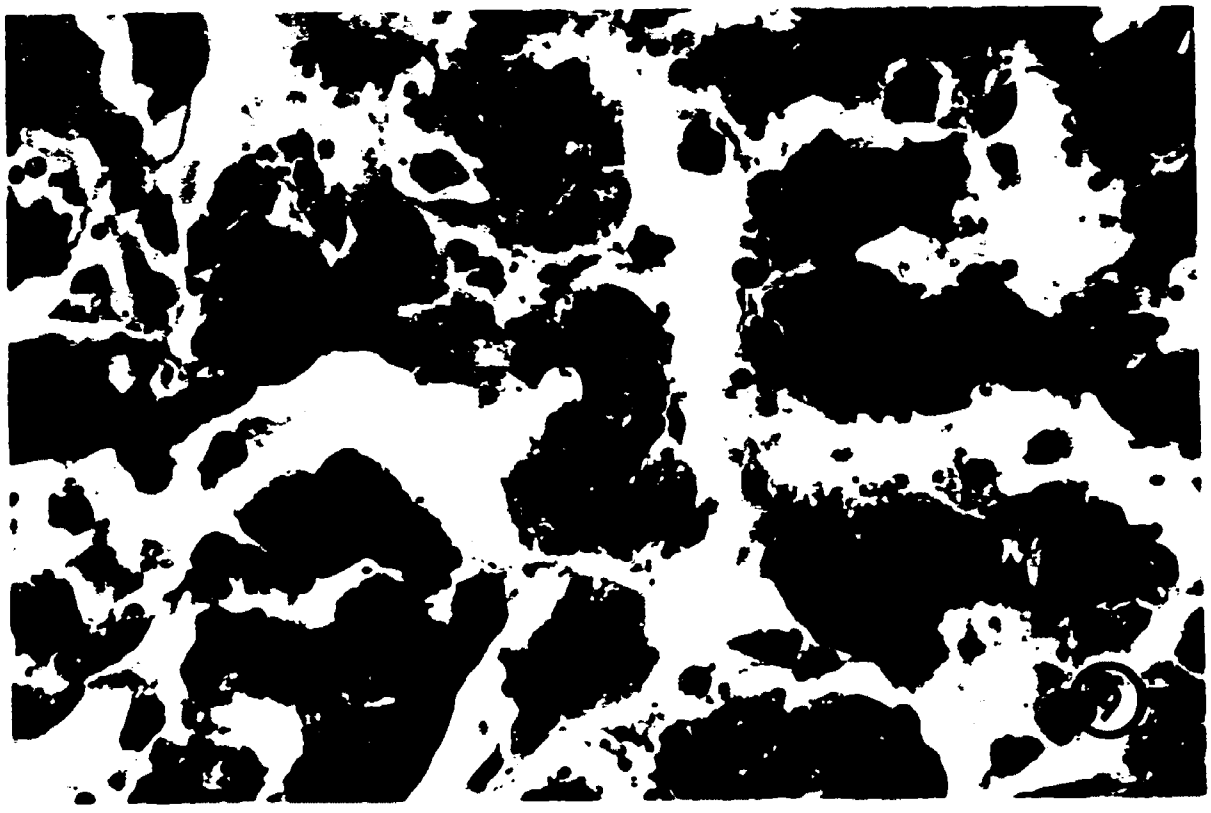
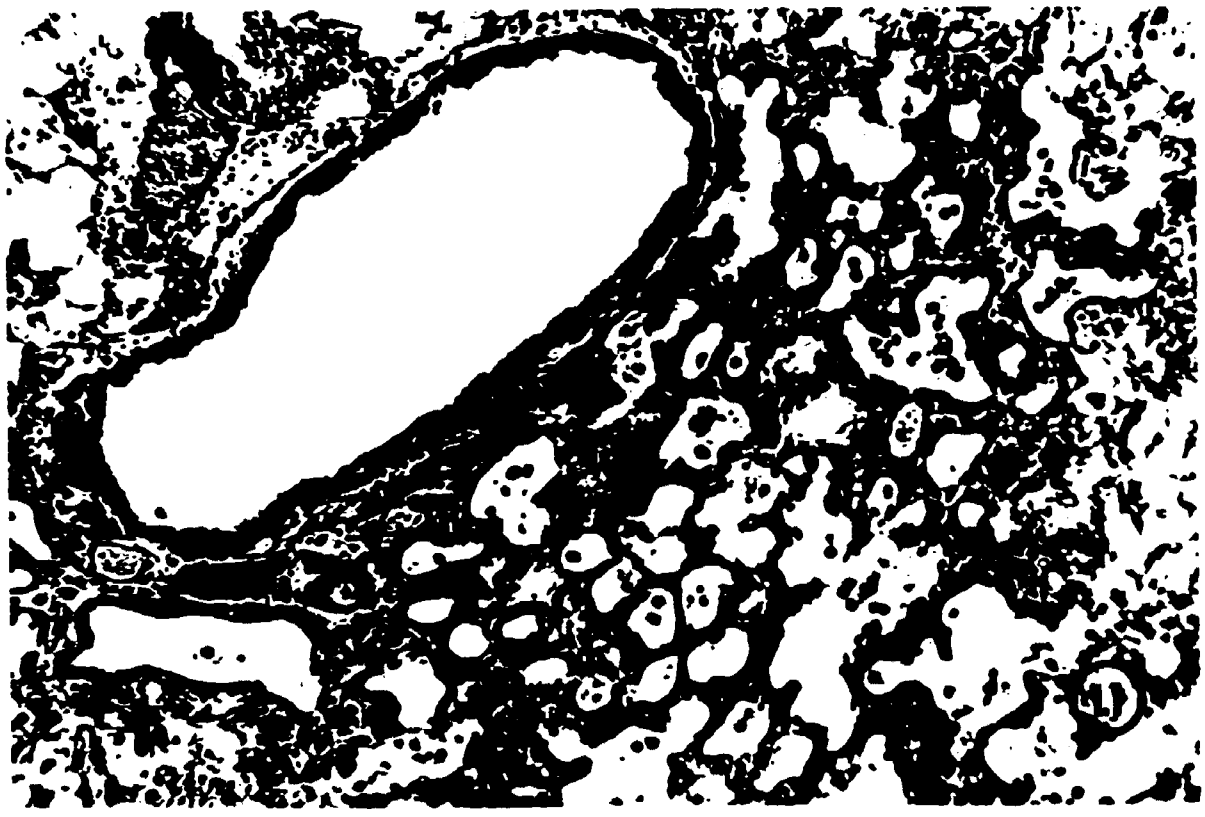


PLATE VI

Figure 11. The early histologic changes in treated mouse lungs are shown. The bronchus is devoid of inflammatory exudate but the surrounding alveolar walls are infiltrated with lymphocytes and plasma cells (1 week of busulfan administration. H&E. 220x).

Figure 12. Atypical pulmonary changes observed in control mouse are demonstrated. There is an infiltration of lymphocytes and plasma cells in the alveolar walls and an accumulation of macrophages in the alveoli (control, 19 weeks. H&E. 220x).



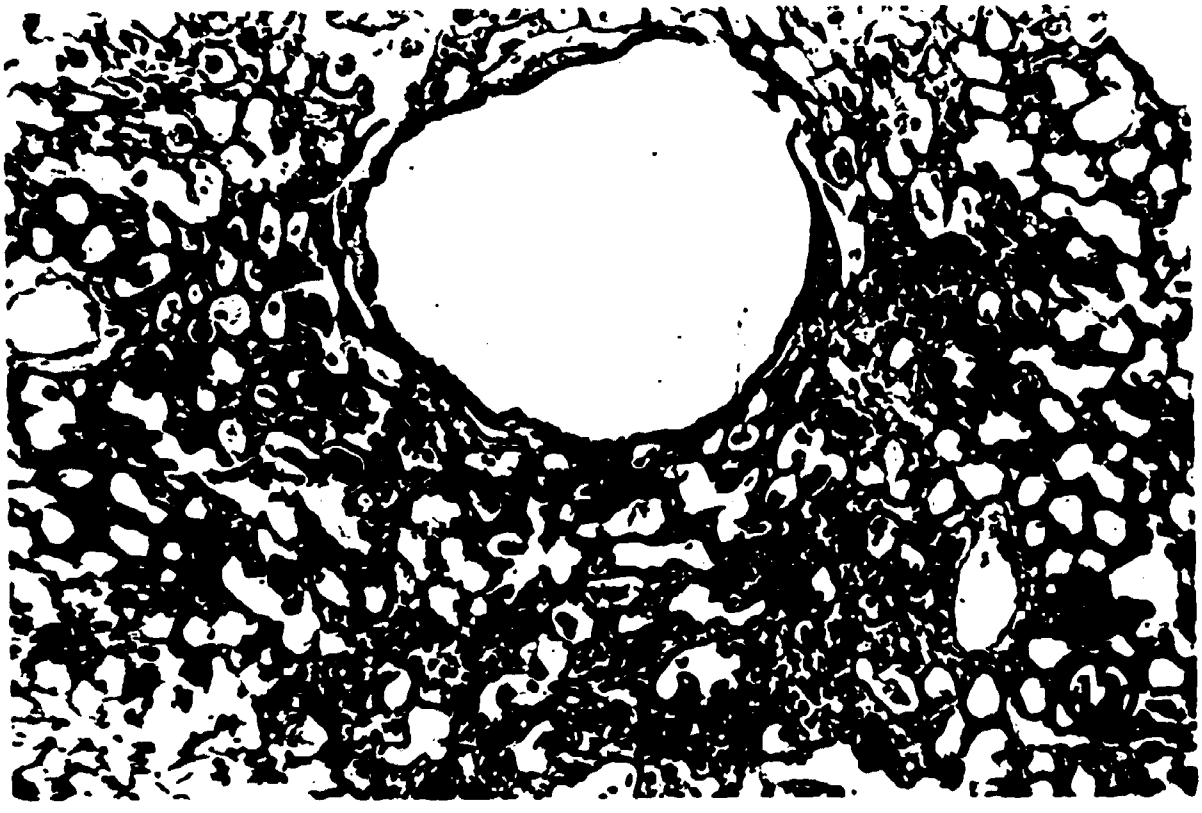
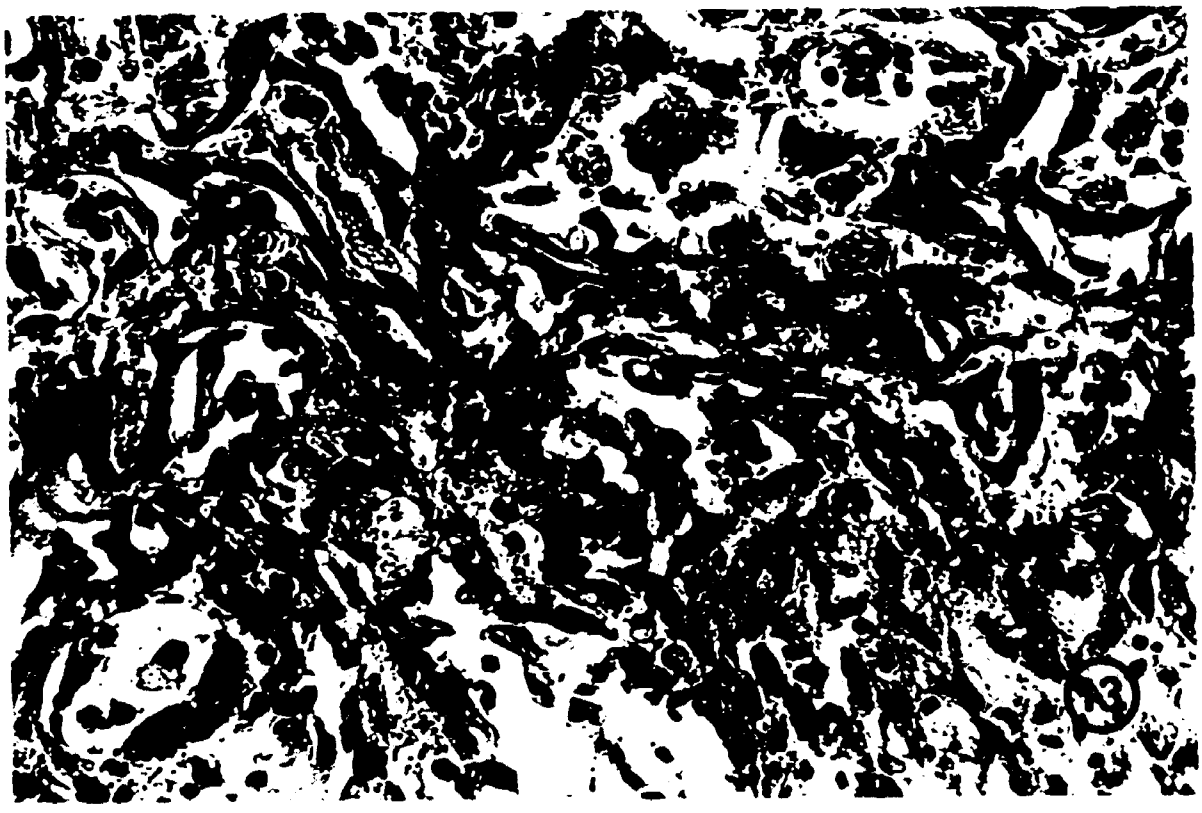


PLATE VII

Figure 13. The alveolar walls are markedly widened due to the proliferation of fibroblasts and connective tissue. A moderate number of mononuclear cells is present (16 weeks of busulfan administration. H&E. 500x).

Figure 14. Another area is shown in which early thickening of the alveolar walls is present. An infiltration of polymorphonuclear leukocytes, mononuclear cells and fibrin is also present (26 weeks of busulfan administration. H&E. 220x).



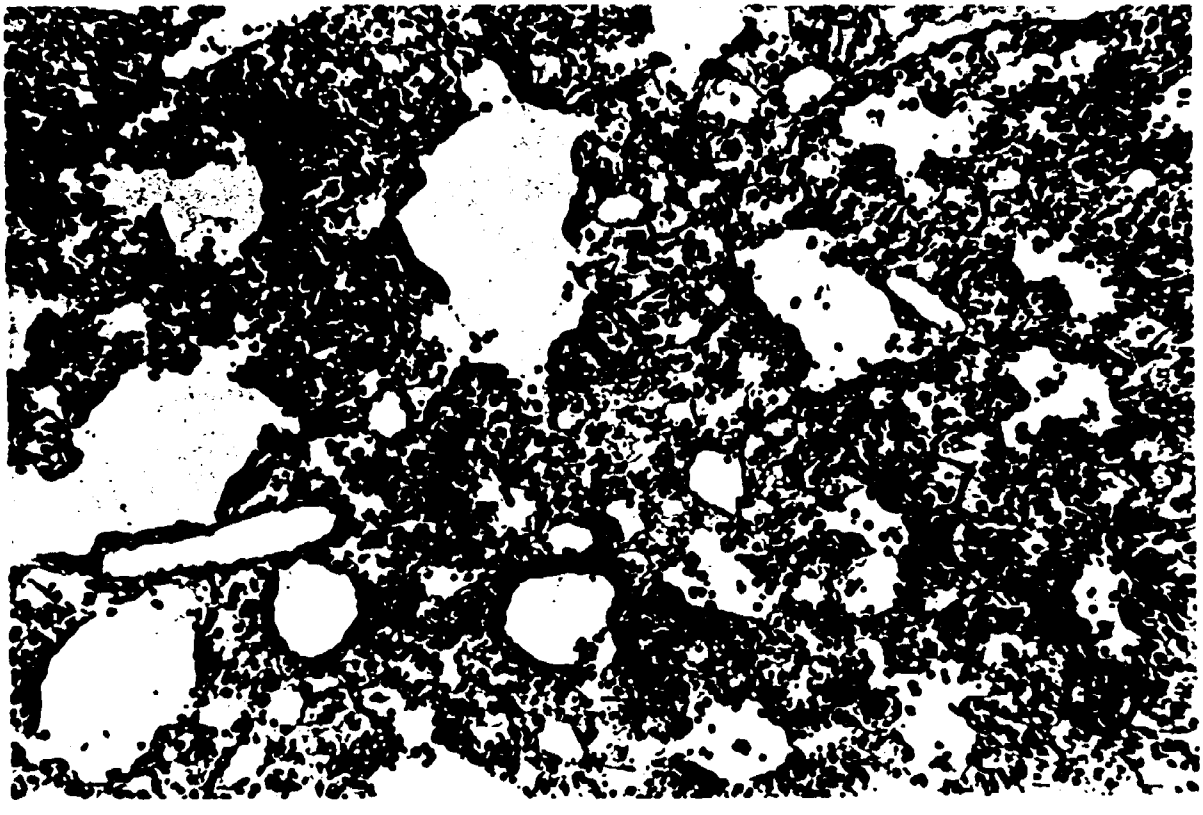


PLATE VIII

Figure 15. In an area of severe fibrosis, the trapped alveoli are lined by giant and multinucleated atypical alveolar cells with hyperchromatic nuclei (15 weeks of busulfan administration. H&E. 500x).

Figure 16. The bronchus is ectatic and plugged with pus cells and necrotic cellular debris. Accumulations of peribronchial and perivascular mononuclear cells are very prominent (14 weeks of busulfan administration. H&E. 220x).

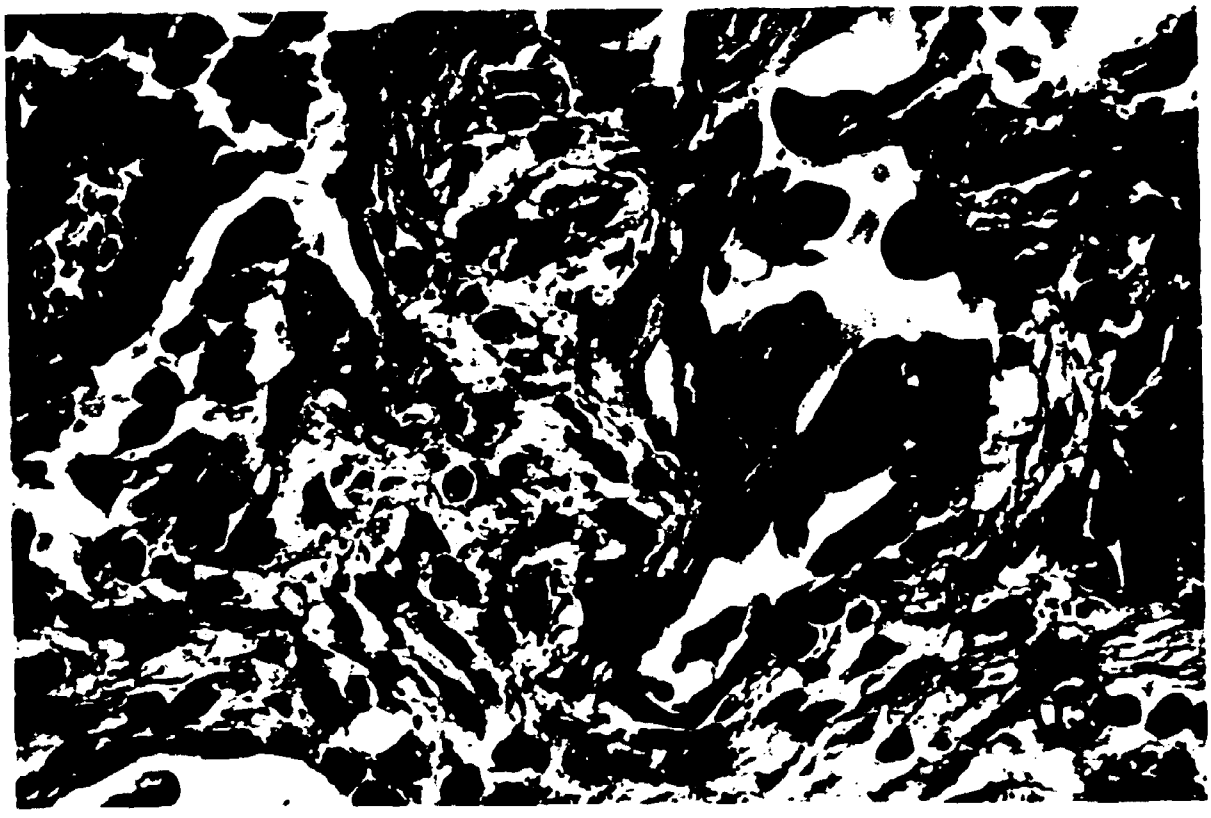
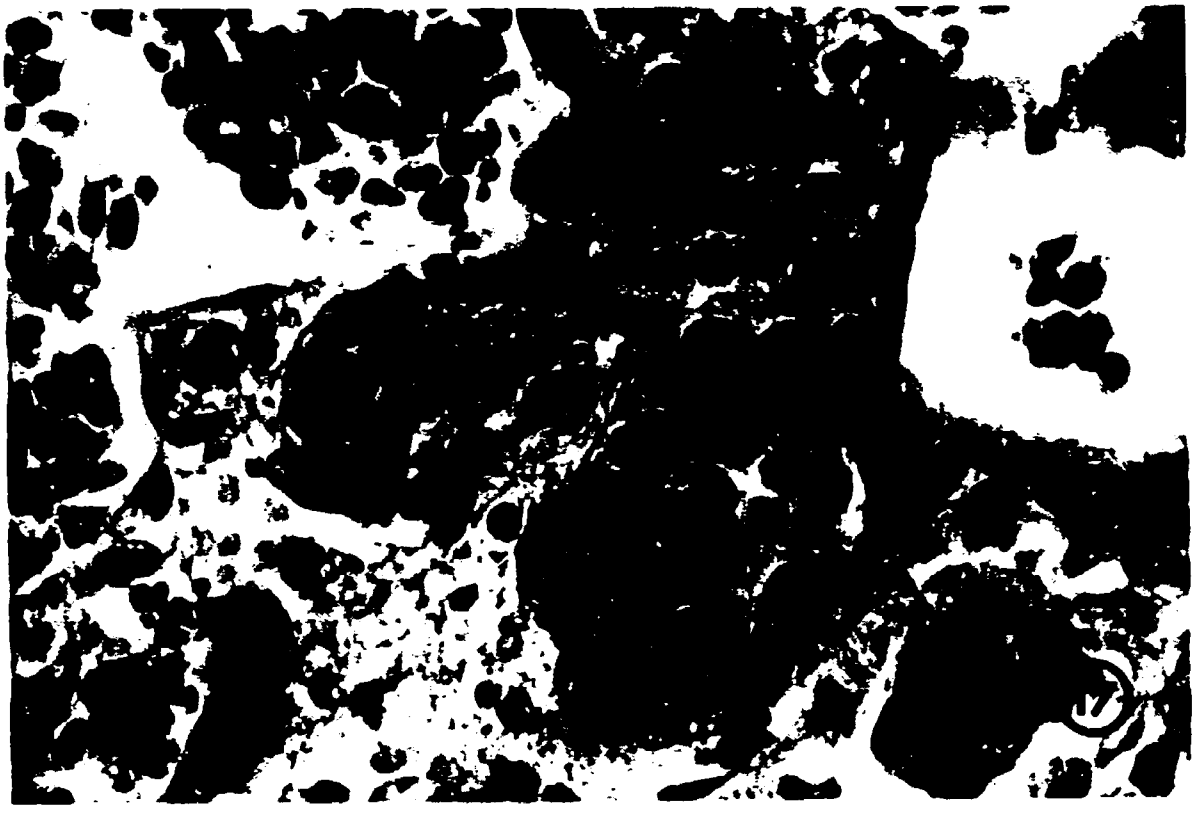


PLATE IX

Figure 17. Bronchial metaplasia is demonstrated. The squamous epithelium simulates the appearance of carcinoma *in situ*, but mitotic figures are not present (15 weeks of busulfan administration. H&E. 500x).

Figure 18. Bronchial hyperplasia is depicted. Note the epithelial cells extending through the basement membrane and muscularis mucosae into adjacent alveoli (15 weeks of busulfan administration. H&E. 500x).



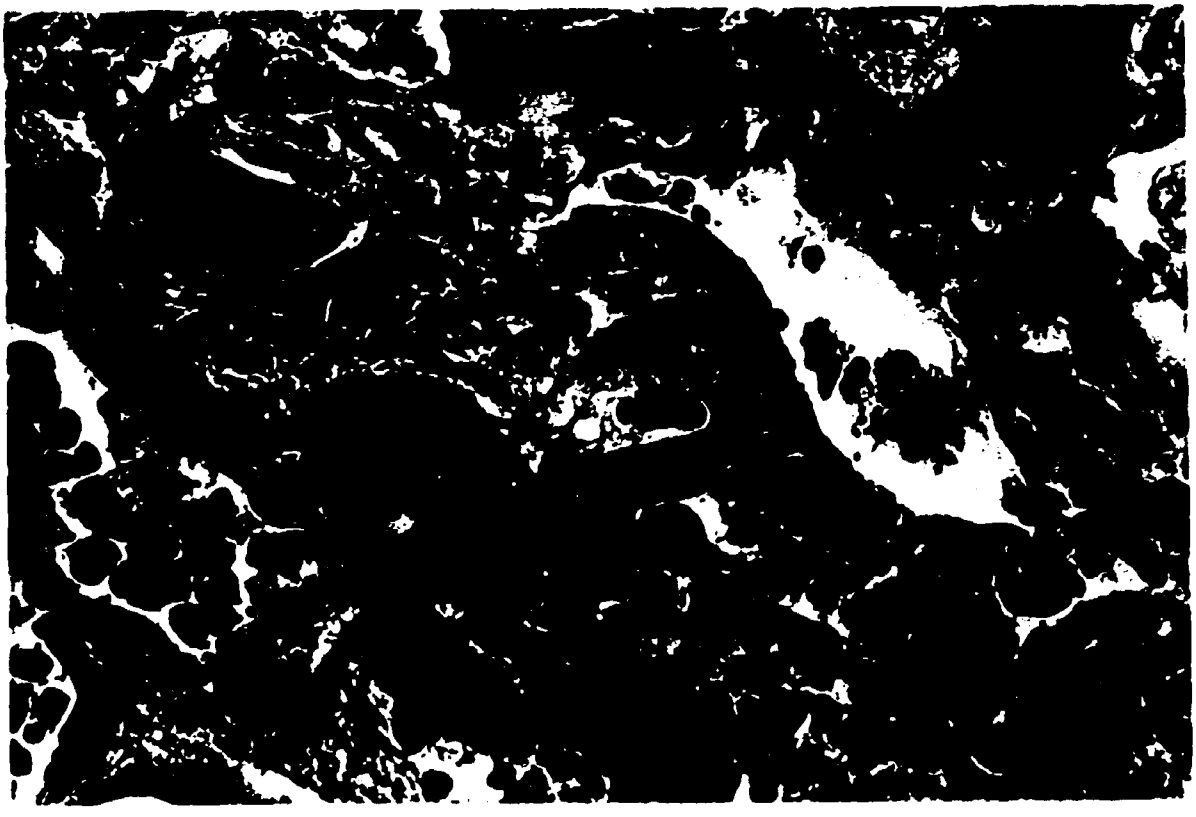


PLATE X

Figure 19. Electron micrograph of bronchial epithelium. Several Mycoplasma pulmonis (MP) are seen interspersed between the microvilli (Mv) of the bronchus (14 weeks of busulfan treatment. Uranyl acetate and lead citrate. 13,600x).

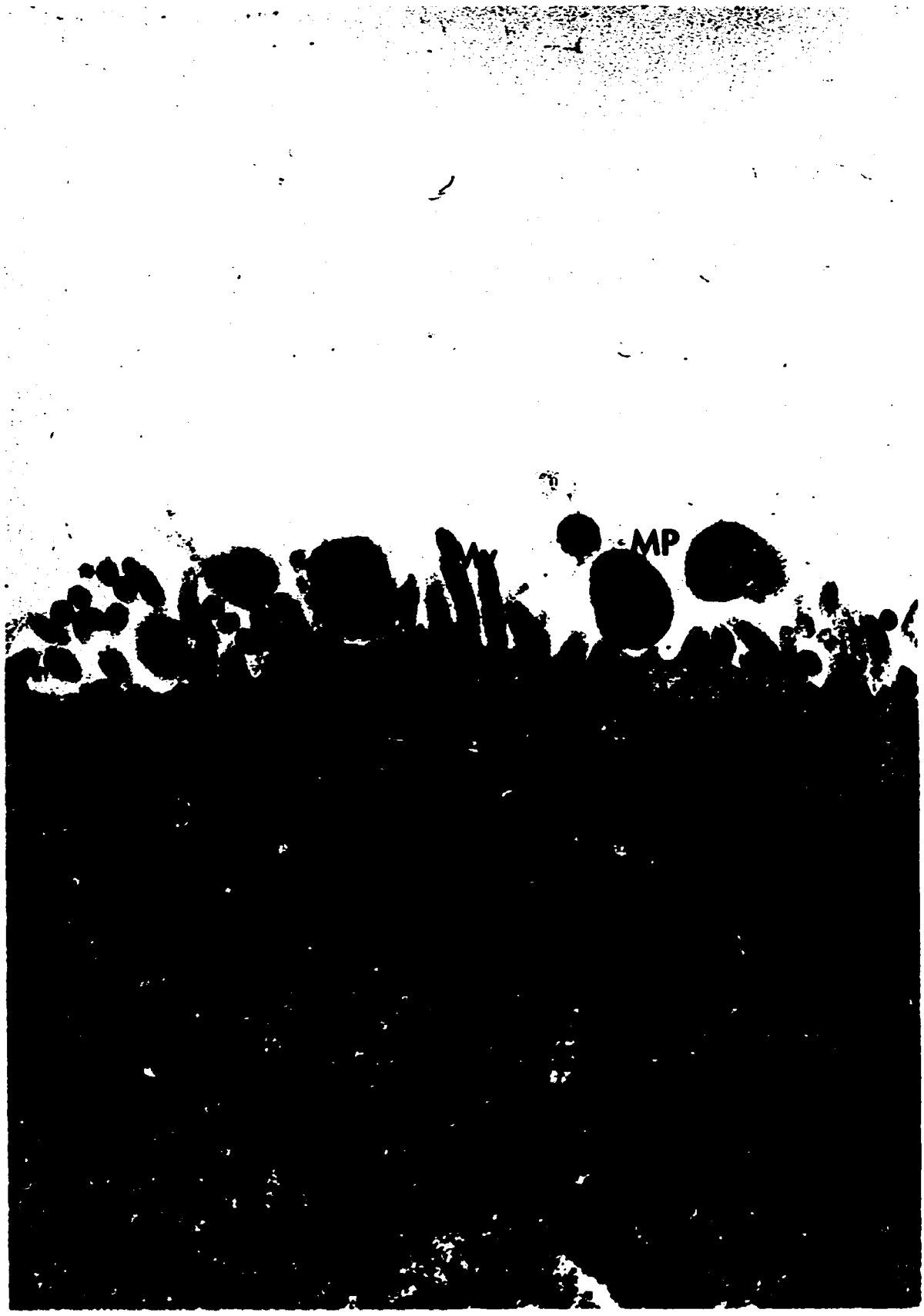

MP

PLATE XI

Figure 20. Electron micrograph of bronchial lumen. The lumen is filled with M pulmonis organisms (MP) and polymorphonuclear leukocytes. Some of the organisms have been phagocytized by the polymorphonuclear leukocyte (arrows) (21 weeks of busulfan treatment. UA & LC. 13,600x).

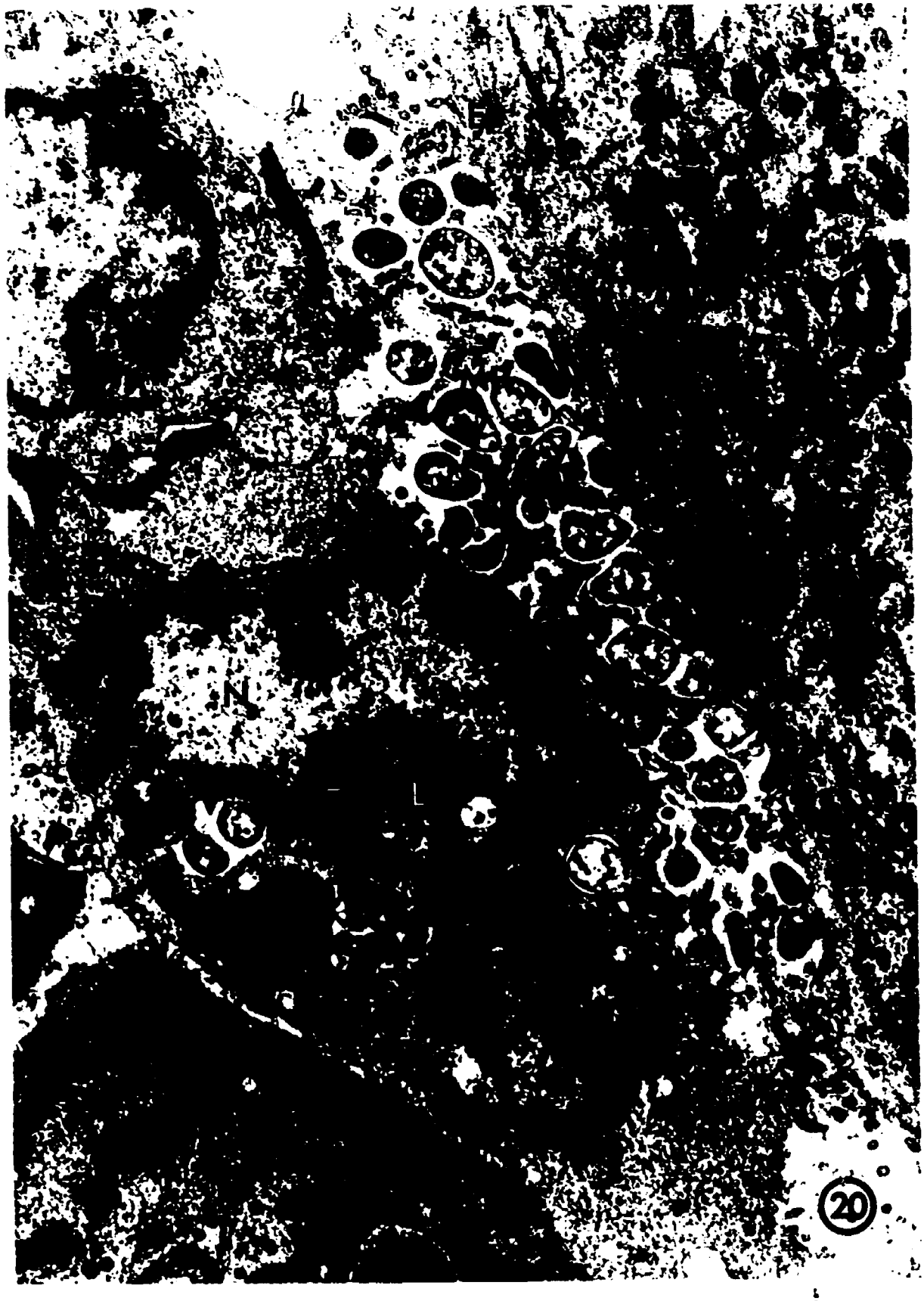

N
L
20

PLATE XII

Figure 21. Bronchial epithelium from a control mouse which shows that the epithelial surface is free from M pulmonis organisms (23 weeks, control. UA & LC. 6,600x).

PLATE XIII

Figure 22. Section of alveolar spaces showing infiltration of polymorphonuclear leukocytes (Poly), alveolar macrophages (Mac) and desquamated type II pneumocytes (AC II) (23 weeks of busulfan treatment. UA & LC. 5,700x).

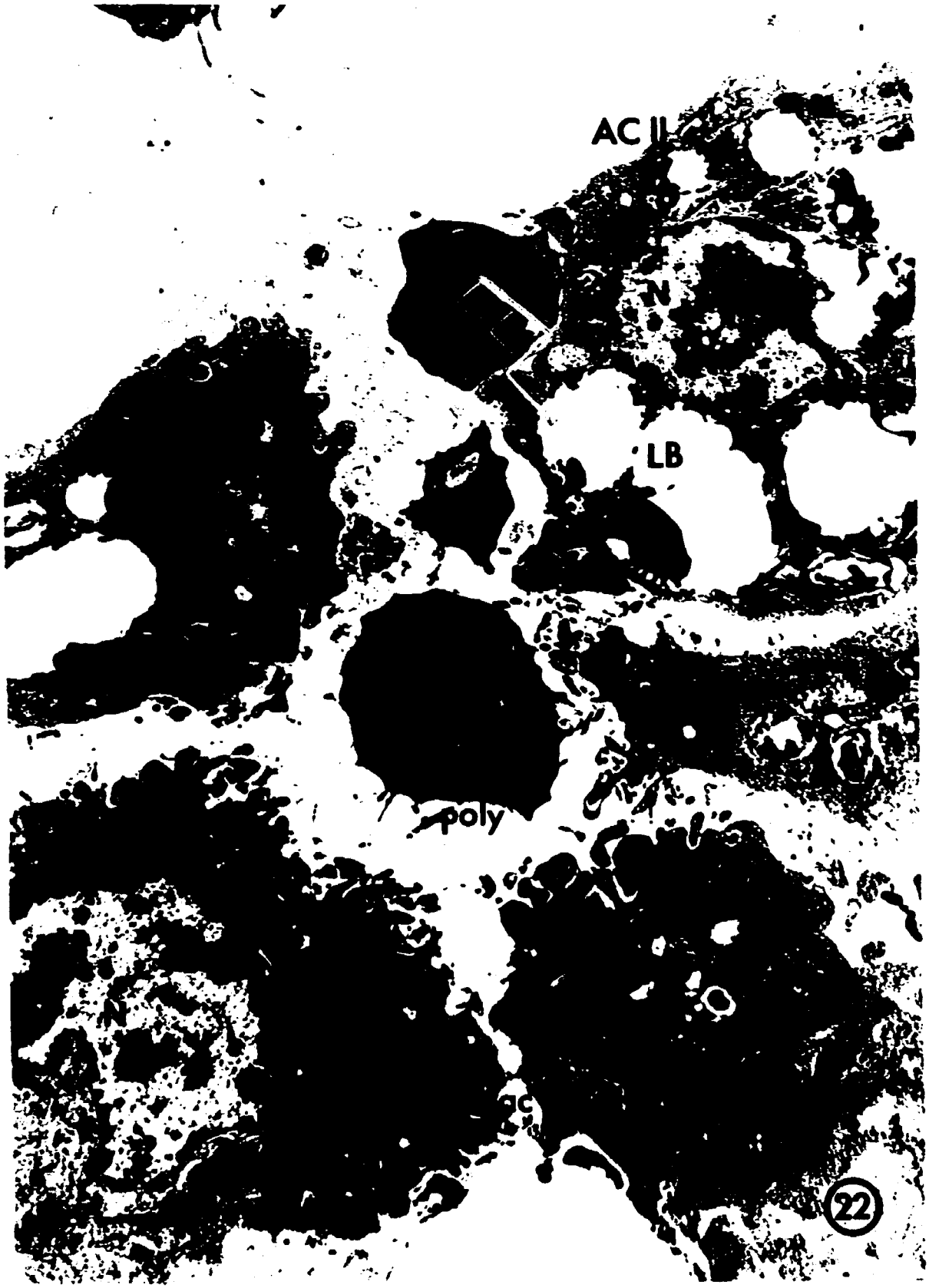

AC II
N
LB
poly
gc
22

PLATE XIV

Figure 23. Alveolar macrophage. Note the numerous crystal-like spicules (CS) within the cytoplasm of the macrophage (15 weeks of busulfan treatment. UA & LC. 18,500x).


23

PLATE XV

Figure 24. Alveolar lining epithelium. Hyperplasia of alveolar type II cells (AC II) with characteristic osmiophilic lamellar bodies (LB) is very prominent in busulfan-treated mice (25 weeks of busulfan treatment. UA & LC. 3,900x).

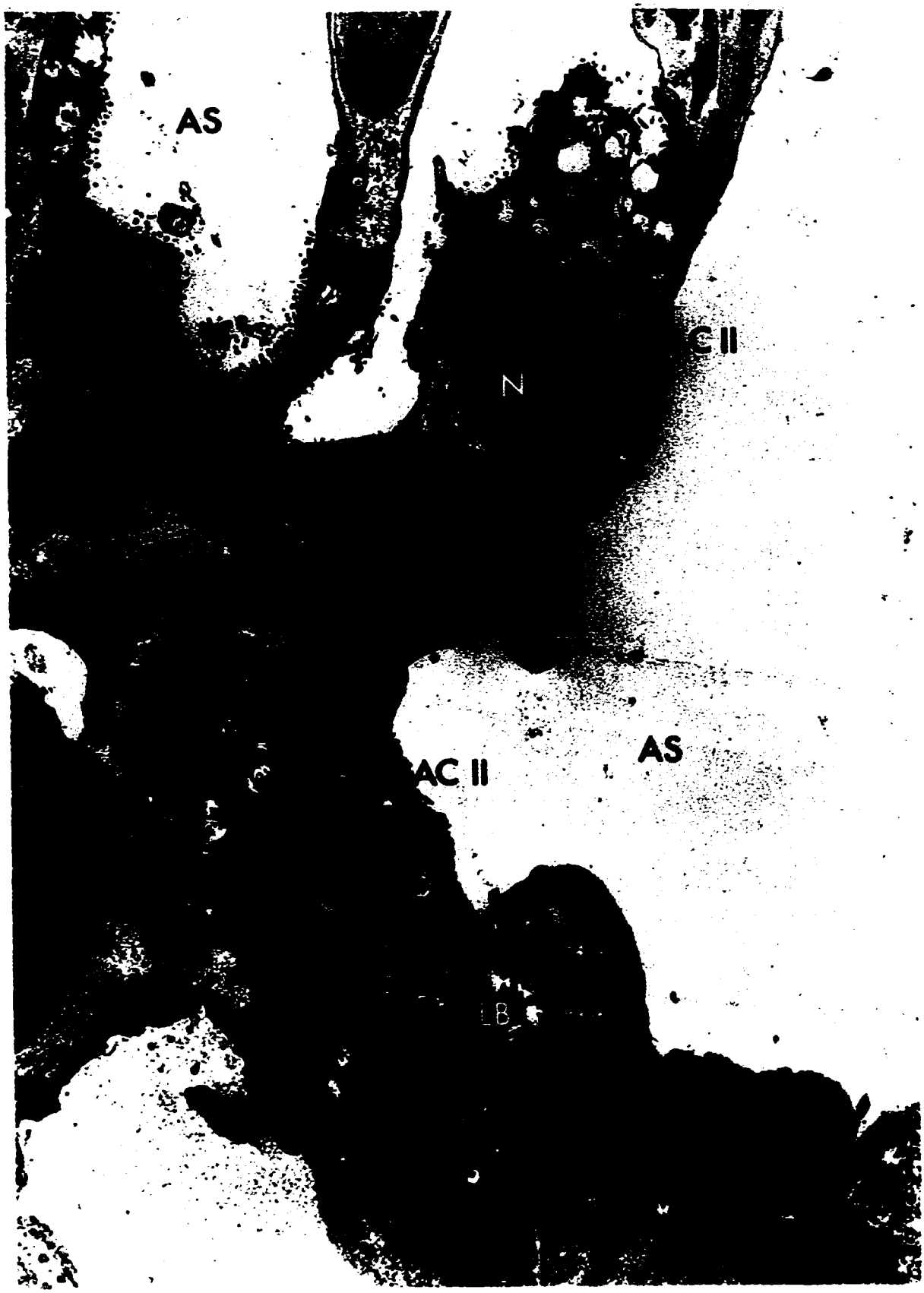

AS
C II
N
AC II
AS
LB

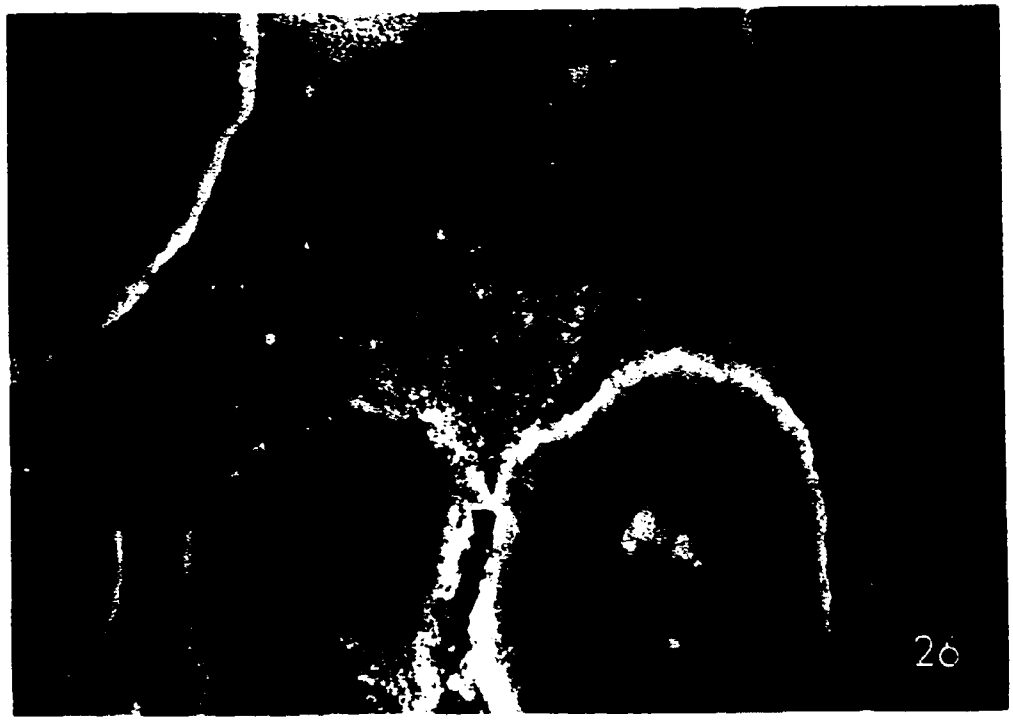
PLATE XVI

Figure 25. Alveolar wall. Note the markedly increased collagen fibrils (CF) in the septal interstitium (23 weeks of busulfan treatment. UA & LC. 12,000x).

(25)

PLATE XVII

Figure 26. A hyperplastic bronchus in busulfan-administered mouse treated with fluoresceinated antibody against M pulmonis is shown. Note the bright white band of fluorescence appearing as a distinct zone along the surface of bronchial epithelium (450x).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