CONF-790249--1

MASTER

A PATHOGENIC MECHANISM IN LUNG FIBROSIS

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Research sponsored by the Office of Health and Environmental Research, U. S. Department of Energy, under contract W-7504-eng-26 with the Union Carbide Corporation.

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Synopsis

The purpose of the study was to examine whether an interaction between two agents causing alveolar epithelial damage would produce lung fibrosis. In mouse lung, intraperitoneal injection of the anti-oxidant butylated hydroxytoluene causes diffuse alveolar type I cell necrosis, followed by proliferation of type II alveolar cells. In animals exposed to 70% O_2 or 100-200 rad X-rays during the phase of type II cell proliferation following BHT, diffuse interstitial lung fibrosis developed within 2 weeks. Quantitative analysis of the lungs for hydroxyproline showed that the interaction between BHT and O_2 or X-rays was synergistic. If exposure to O_2 or X-rays was delayed until epithelial recovery was complete, no fibrosis was seen. Abnormally high levels of lung collagen persisted up to 6 months after one single treatment with BHT and 100 rad X-rays. A commonly seen form of chronic lung damage may thus be caused by an acute interaction between a bloodborne agent which damages the alveolar cell and a toxic inhalant or X rays, provided a critically ordered sequence of exposure is observed.

Pathogenetic features common to many forms of interstitial pulmonary fibrosis are a chronic alveolitis, changes in the cellular composition of the alveolar zone and derangement of the interstitial collagen. Fibrosis often appears to develop as a common sequel to injury when normal tissue repair fails to take place.[1] It may be precipitated and sustained by exposure to a single etiologic agent such as inhaled particles of silica or irradiation of the thorax. In other forms, such as idiopathic pulmonary fibrosis, the etiologic agent remains unknown. Recently we suggested that pulmonary fibrosis could develop following the interaction between an agent reaching the lung via the bloodstream and a toxic inhalant.[2] This speculation was based upon the following experimental observations.

In mice, the antioxidant butylated hydroxytoluene (BHT) causes widespread and uniform lung damage. Twenty-four hours after an intraperitoneal injection or oral administration of 200-400 mg/kg of BHT, diffuse necrosis of type I alveolar epithelial cells is seen throughout the lung. The initial damage is followed by a period of recovery. On days 2 and 3 after BHT there is intensive cell proliferation in lung, and total pulmonary DNA synthesis, measured by incorporation of labelled thymidine into pulmonary DNA, increases 10-15 fold.[3] In the early phase of recovery, most dividing cells are type II epithelial cells. From days 5 to 6 lesions develop in some capillary endothelial cells and endothelial cell proliferation follows. Interstitial cells appear not to be damaged, but also proliferate at this time. Six to 10 days after a single injection of BHT, the lungs regain a virtually

normal appearance. The morphologic sequence of events following BHT has been fully documented both by light- and electron microscopy.[4,5]

We subsequently examined the effects of O_2 exposure (60 to 100% for 10 to 24 hrs) upon lung cell division on different days after BHT. We found that O_2 inhibited cell division 2, 3 and 4 days after BHT, but no longer on days 5, 6 and 7.[6] Since the early phase after BHT is characterized by primarily division of type II alveolar cells, whereas interstitial cells divide on days 5 through 9 after BHT[4] we concluded that dividing epithelial cells might be more susceptible to the cytotoxic action of O_2 than dividing interstitial cells. Selective killing of epithelial cells by early oxygen exposure after BHT-induced lung injury could then allow excessive proliferation of interstitial cells and lead to the development of fibrosis. Experiments described in this paper were designed to test this hypothesis further.

Methods

Young adult male BALB/c mice, weighing 20-25 g, were injected i.p. with a single dose of 400 mg/kg of BHT (3-5-di-tert-butyl-4-hydroxytoluene), dissolved in corn oil. Control animals received corn oil alone (0.1 ml/10g). Exposure to O_2 was performed in a plexiglass chamber, ventilated with a humidified mixture of O_2 and compressed air; the O_2 concentration was periodically monitored with an oxygen analyzer and kept within ±3% of the desired concentration. Local irradiation of the thorax was done with a GE Maxitron 300 X-ray machine operated at 300 kVp and 20 mA. The HVL was 1.29 cm Cu. The average dose rate was 265 rads/min.

Detailed experimental protocols are given in the results section.

At the end of the experiments, animals were anesthetized with sodium pentobarbital and exsanguinated via the abdominal aorta; the lungs were fixed in situ with 10% neutral buffered formalin injected through the trachea. Lung lobes were embedded in paraffin, sectioned at 3-4 μm and stained with hematoxylin and eosin, Masson's trichrome, van Gieson's and Snook's reticulin stains. Collagen was quantitated chemically in another group of mice by analyzing the lungs for hydroxyproline content. The lungs were perfused in situ with 0.9% NaCl, excised, lyophilized and hydrolyzed in 6 N HCl for 18 hrs at 107°C. Hydroxyproline was determined by a colorimetric assay[7] and all results were calculated as μg hydroxyproline per total lung. One-way analysis of variance was performed and a p value of < 0.05 was considered significant; comparisons of means were done by using Student's t-test. More details on methodological procedures have been given elsewhere.[2,8]

Results

The first experiment was designed to examine the interaction between BHT and O_2. A group of mice received 400 mg/kg of BHT i.p. Half of the group was placed into 70% O_2 immediately after BHT and removed from the chamber 6 days later. The other half was kept in room air for 7 days and then placed in 70% O_2 for 6 days. Control groups were animals injected with corn oil, kept either in O_2 or in room air, and animals given 400 mg/kg of BHT and kept in room air. All animals were

killed for histopathologic analysis of the lung and determination of hydroxyproline two weeks after the BHT injection.

In oil treated control mice, total lung hydroxyproline varied between 200 and 230 µg/total lung, corresponding to 7.4-8.5 mg/g dry weight (Table 1). Exposure of oil-treated animals for 6 days to 70% O_2 alone was without any substantial effect upon total lung hydroxyproline. Administration of 400 mg/kg of BHT produced, within 2 weeks, a small increase in total pulmonary hydroxyproline. However, a much larger increase in total lung hydroxyproline, 150% over values found in the oil-treated control group, was found in animals given BHT and placed immediately for 6 days into 70% O_2. Since O_2 treatment alone had at best only a marginal effect and since BHT alone raised lung hydroxyproline only 50% above levels found in oil-treated controls the combined action of BHT and O_2 was not only additive, but synergistic. Histopathological examination showed that lungs from animals injected with oil and exposed to 70% O_2 were indistinguishable from the lungs of control animals kept in air. In animals treated with BHT, the acute mild pneumonitis seen 2-6 days after injection[4] was virtually resolved within 2 weeks and only focal alveolar wall hypercellularity and occasional intraalveolar macrophages remained (Figure 1). This was in sharp contrast to the lesions present in animals 2 weeks after treatment with BHT and immediate exposure for 6 days to O_2. Pulmonary architecture was severely disrupted due to cellular infiltration, primarily interstitial, and focal consolidation. There was a marked increase in interstitial cells or fibroblasts and in the amount of fibrillar material present within alveolar septa (Figure 2).

Special stains showed that the fibrillar material was positive for collagen and that there was a marked increase in reticulin fibers of variable length and thickness in the alveolar septa (Figure 3). The histopathological observations were thus in full agreement with the biochemical data.

On the other hand, when exposure to 70% O_2 was delayed until the 7th day after BHT, little fibrosis was observed histologically and the total lung hydroxyproline value was similar to the one found in animals treated with BHT alone (Table 1).

In the next experiment, animals were injected with BHT and placed immediately in 70% O_2 for 4 days. They were returned to room air and killed 2 weeks after BHT. Results in Table 2 show that a 4 day exposure to O_2 was almost as efficient as a 6 day exposure. If we waited until 3 days after BHT before placing the animals for 4 days into O_2, total pulmonary hydroxyproline content was still higher than in the lungs of animals treated with BHT alone, but somewhat lower than in the group placed immediately after BHT into O_2 (Table 2).

Another way to interfere with the epithelial recovery following BHT induced lung injury was to irradiate the lung with X-rays instead of exposing it to O_2. The data of this experiment are given in Table 3. Irradiation of control animals with 200 rad did not produce abnormal hydroxyproline accumulation in the lung within 2 weeks, and BHT treatment alone had only a slight effect. However, if 200 rad were delivered to the thorax one day after BHT, diffuse interstitial fibrosis developed within one to two weeks as determined by histological and

biochemical procedures. Delay of thorax irradiation until 6 days after BHT, on the other hand, was without any effect.

It was of interest to determine whether the increased levels of pulmonary hydroxyproline following the acute interaction between BHT and X-rays would persist for a prolonged time period. Data of such an experiment are given in Table 4. Significantly increased levels of total lung hydroxyproline were still present 6 months after an initial treatment with BHT and 100 rad given one day later. In contrast to the persistence of markedly elevated lung hydroxyproline levels, histological examination showed marked regression of lesions over the 24 week period. At two weeks there was marked interstitial pneumonitis (Figure 4) with focal consolidation, fibroblastic proliferation and an increase in reticulin fibers on Snook's reticulin stain. Consolidation was most frequently subpleural and associated with partial parenchymal collapse adjacent to ectacic bronchioles. By 24 weeks the inflammatory component of the lesion had virtually disappeared leaving behind slightly thickened and hypercellular alveolar septa with indistinct borders (Figure 5). Persistence of the increased number of reticulin fibers could be detected with Snook's reticulin stain. Focal subpleural consolidation and parenchymal collapse also persisted.

Discussion

The data presented in this paper show that it is possible to produce an abnormal and persistent accumulation of collagen in mouse lung by combining two treatments: first, administration of BHT, a

bloodborne agent which causes diffuse and uniform necrosis of the type I alveolar epithelial cells, followed by a treatment (exposure to 70% O_2 or low doses of X-rays) which inhibits or prevents cell division in lung. The developping lesions are indicative of diffuse interstitial fibrosis. Preliminary ultrastructural observations suggest that this model has many features in common with the Hammon-Rich syndrome in man (Brody, A., personal communication).

Quantitative determination of lung hydroxyproline shows that BHT alone causes a significant increase in collagen, whereas neither 70% O_2 nor 200 rad X-rays are sufficient to produce fibrosis. If BHT is combined with either O_2 exposure or thorax irradiation the total amount of hydroxyproline found in lung now exceeds by far the amount which accumulates after BHT alone. The two insults to the lung have thus a synergistic effect. However, synergism is only seen if a strict temporal relationship between the two exposures is maintained. Oxygen exposure or thorax irradiation must occur within the first few days following BHT injection. If O_2 exposure or thorax irradiation is delayed for 6 days there is no abnormal accumulation of lung collagen and no histopathological evidence of interstitial fibrosis. There is also no fibrosis if O_2 or X-rays are administered prior to BHT.[2,8]

The mechanism underlying this interaction between BHT and O_2 or X-rays in causing lung fibrosis has not been fully elucidated. At present, we explain our findings as follows: following the initial lung damage, there is first a proliferation of epithelial cells. If O_2 or X-rays interfere with this phase of epithelial cell proliferation

which is essential for reestablishment of a normal alveolar surface, the interstitial cell population could begin to grow comparatively uninhibited. In support of this hypothesis is the observation made in another experimental model: if an excised trachea is stripped of its epithelium and implanted subcutaneously into a syngeneic host, the tracheal lumen will become obliterated with connective tissue within a very short time. However, if isolated epithelial cells are reintroduced into the tracheal lumen, reepithelizsation follows and the trachea remains open.[9] In trachea, fibroblasts grow thus excessively in the absence of an intact epithelium. It is tempting to speculate that the proliferation of interstitial cells in the alveolar zone is controlled, directly or indirectly, by the continuous presence of an intact epithelial layer.

It will be important to examine in future studies whether we can produce a similar interaction between agents other than BHT and O_2 or X-rays, and then come to a general conclusion about the pathogenetic principle underlying the development of at least some forms of lung fibrosis. Diffuse damage to the alveolar zone may be caused by many toxic inhalants or by several bloodborne agents.[10] It is also conceivable that the same or other agents might interfere with epithelial recovery following the initial injury. A commonly seen form of chronic lung damage might thus be caused by an acute synergistic interaction between two agents, provided a critically ordered sequence of exposure takes place.

There are two specific clinical situations where it is conceivable that the development of fibrotic lung changes might be enhanced by

a synergism similar to the one seen in our animal studies. Adequate treatment of critically ill patients often requires O_2 therapy. It is however often difficult to decide on the concentration of O_2 and the duration of treatment which will not cause lung damage. Many patients will suffer from adult respiratory distress syndrome and whether the lesion develops can often not be predicted.[11] Since trauma, shock, fatty embolism and many drugs can cause acute alveolar damage[12] it is possible that development of pulmonary complications during O_2 therapy is determined by the presence and severity of initial lung damage rather than by the O_2 treatment. If this can be substantiated, it will become necessary to devise appropriate diagnostic tests which would allow detection of alveolar epithelial cell damage so that patients at risk can be identified.

Lung fibrosis can also develop within a few weeks in patients treated with irradiation to the thorax and given concomittantly antineoplastic agents such as bleomycin, cyclophosphamide, actinomycin D or others.[13] Cytotoxic agents are known to cause acute alveolar cell death. It is feasible that the accelerated development of radiation-induced lung fibrosis is caused by a mechanism similar to the one found in the study of BHT and X-ray interaction. Based on these findings, timing between drug administration and chest irradiation might be an important factor in determining whether excessive fibrosis develops.

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Figure 1.

Lungs from mice injected i.p. with 400 mg/kg BHT, kept in air, and killed 2 weeks after BHT. Hypercellularity of the alveolar septa and occasional intraalveolar macrophages are present. H & E x 200.

Figure 2.

Lungs from mice injected i.p. with 400 mg/kg BHT, immediately exposed to 70% oxygen for 6 days, and killed 2 weeks after BHT. Disruption of normal parenchymal architecture is due to cellular infiltration and consolidation. H & E X 200.

Figure 3.

Lungs from mice injected i.p. with 400 mg/kg BHT, immediately exposed to 70% oxygen for 6 days, and killed 2 weeks after BHT. Large numbers of reticulin fibers of varying thickness are present in alvolar septa and area of consolidation. Snook's reticulin stain X 500.

Figure 4.

Lungs from mice injected i.p. with 400 mg/kg BHT, irradiated with 100 rad X-ray one day later, and killed 2 weeks after BHT. Interstitial pneumonitis characterized by hypercellularity and thickening of alveolar walls is present. H & E X 250.

Figure 5.

Lungs from mice injected i.p. with 400 mg/kg BHT, irradiated with 100 rad X-ray one day later, and killed 24 weeks after BHT. Alveolar walls are slightly thickened and hypercellular with indistinct borders. H & E X 250.

Table 1: Total lung hydroxyproline in mice exposed to 70% O_2 following BHT[a]

Treatment[b]	Hydroxyproline per lung (µg)	
	Oxygen exposure on days 1-6 after BHT[c]	Oxygen exposure on days 7-12 after BHT[d]
Corn oil + air	206 ± 5	226 ± 8
Corn oil + 70% O_2	228 ± 4[e]	220 ± 3
BHT + air	296 ± 21[e]	307 ± 19[e]
BHT + 70% O_2	539 ± 19[e,f]	321 ± 14[e]

[a]Data from reference 8; values are means ± SE from 8-10 animals/group

[b]Male mice received BHT (400 mg/kg) i.p. or corn oil (0.1 ml/10g) i.p. and were killed 2 weeks later.

[c]Exposure to 70% O_2 begun immediately after BHT injection.

[d]Exposure to 70% O_2 begun 7 days after BHT injection.

[e]$p < 0.05$ compared to animals injected with corn oil and kept in air.

[f]$p < 0.05$ compared to animals injected with BHT and kept in air.

Table 2: Total lung hydroxyproline in animals exposed to 70% O_2 on different days after BHT

Treatment[a]	Hydroxyproline per lung (μg)[b]
BHT and 70% O_2 on days 1, 2, 3, 4	421 ± 18[c,d]
BHT and 70% O_2 on days 3, 4, 5, 6	341 ± 12[c]
BHT and air	283 ± 10

[a]Male mice injected with 400 mg/kg of BHT and kept in 70% O_2 as indicated, otherwise in air; all animals killed 2 weeks after BHT.

[b]Mean ± SEM; from 10 animals per group.

[c]$p < 0.05$ compared to animals injected with BHT and kept in air

[d]$p < 0.05$ compared to animals injected with BHT and exposed to O_2 on days 3-6.

Table 3: Total lung hydroxyproline in animals irradiated with 200 rad X-rays on different days after BHT[a]

Treatment[b]	Hydroxyproline per lung (µg)	
	Irradiated 1 day after BHT[c]	Irradiated 6 days after BHT[d]
Corn oil + sham irradiation	178 ± 3	219 ± 4
Corn oil + 200 rad	184 ± 5	215 ± 6
BHT + sham irradiation	259 ± 6[e]	295 ± 16
BHT + 200 rad	371 ± 26[e,f]	284 ± 9

[a]Data from reference 9; values are means ± SE from 10 animals/group

[b]Male mice received BHT (400 mg/kg) i.p. or corn oil (0.1 ml/10g) i.p. and were killed 2 weeks later.

[c]200 rad to the thorax 1 day after BHT.

[d]200 rad to the thorax 6 days after BHT.

[e]$p < 0.05$ compared to animals injected with oil and sham irradiated.

[f]$p < 0.05$ compared to animals injected with BHT and sham irradidated.

Table 4: Total lung hydroxyproline in animals irradiated with 100 rad X-rays 1 day after BHT[a]

Weeks after BHT	Hydroxyproline per lung (µg)			
	BHT + 100 rad	BHT + Sham	Corn oil + 100 rad	Corn oil + Sham
2	371 ± 15(7)[b]	287 ± 7(8)	213 ± 6(8)	223 ± 6(8)
6	358 ± 21(6)[b]	282 ± 5(8)	233 ± 6(7)	233 ± 10(8)
12	404 ± 28(7)[b]	325 ± 10(8)	257 ± 9(8)	278 ± 3(7)
24	387 ± 27(7)[b]	319 ± 12(8)	280 ± 6(8)	248 ± 8(8)

[a]Male mice received BHT (400 mg/kg) or corn oil (0.1 ml/10g) i.p. and were irradiated with 100 rad to the thorax or sham irradiated 1 day later. The animals were killed 2, 6, 12 or 24 weeks after BHT.

[b]$p < 0.05$ compared to animals treated with BHT and sham irradiated.

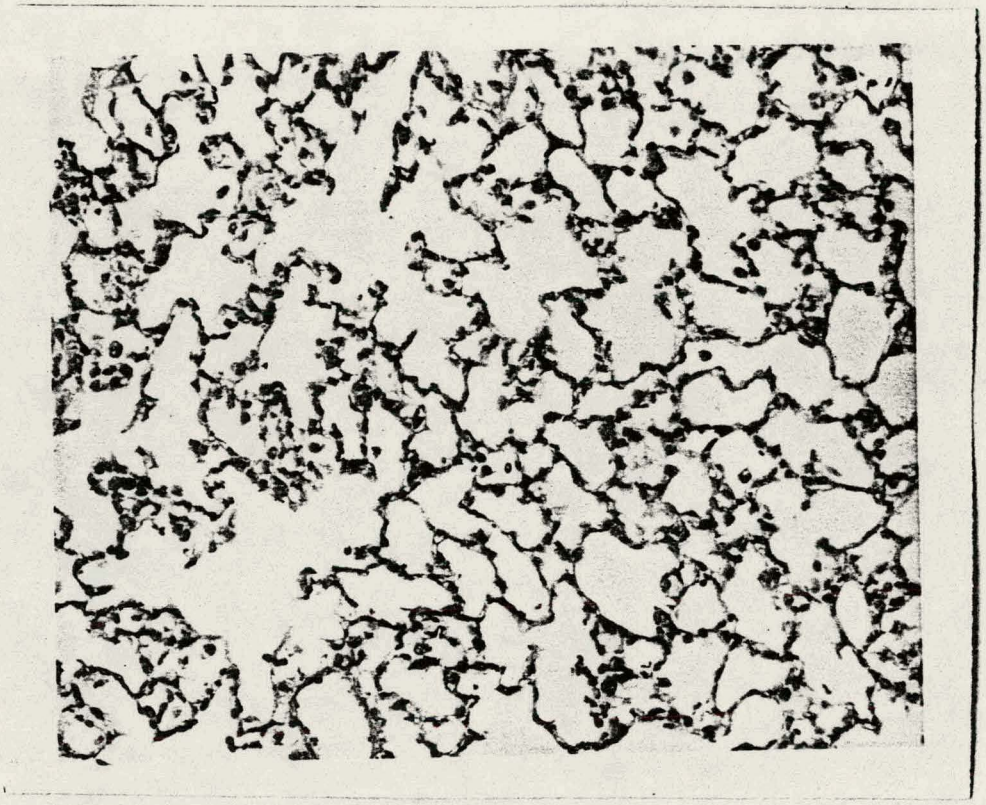

fig 1

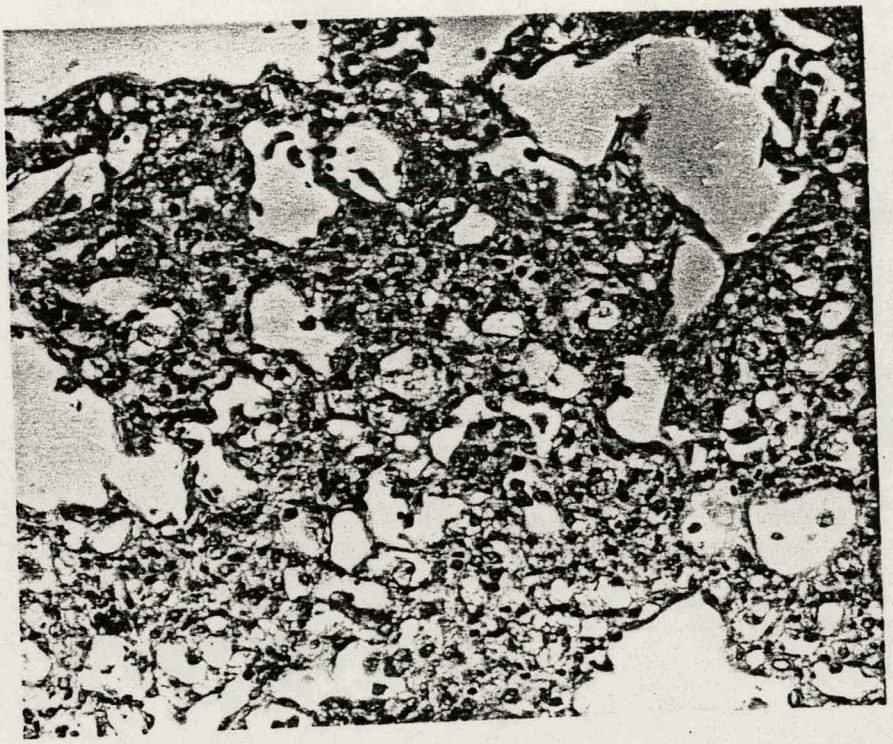

fig 2

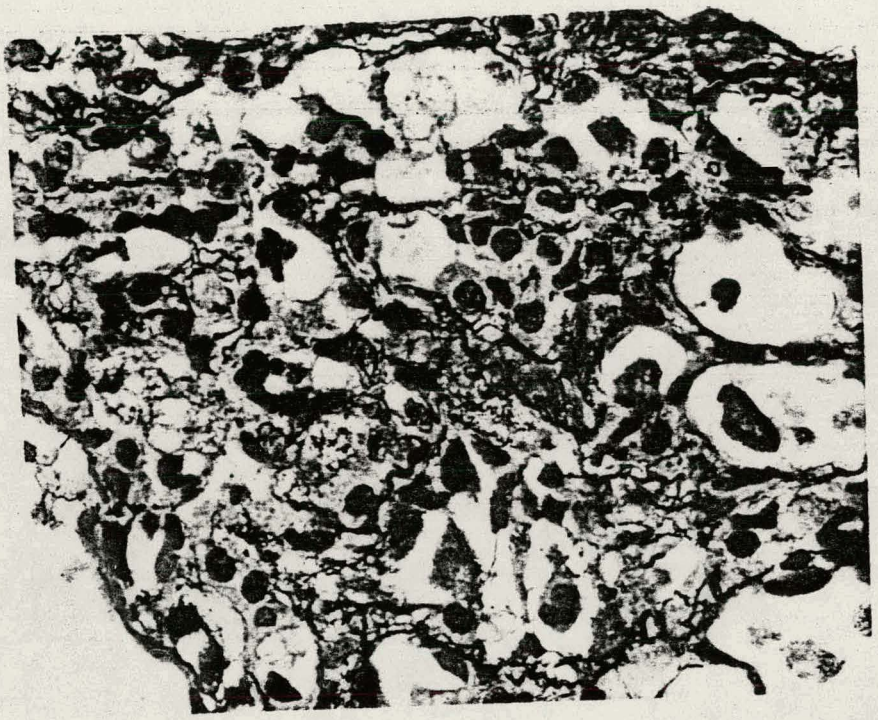

fig 3

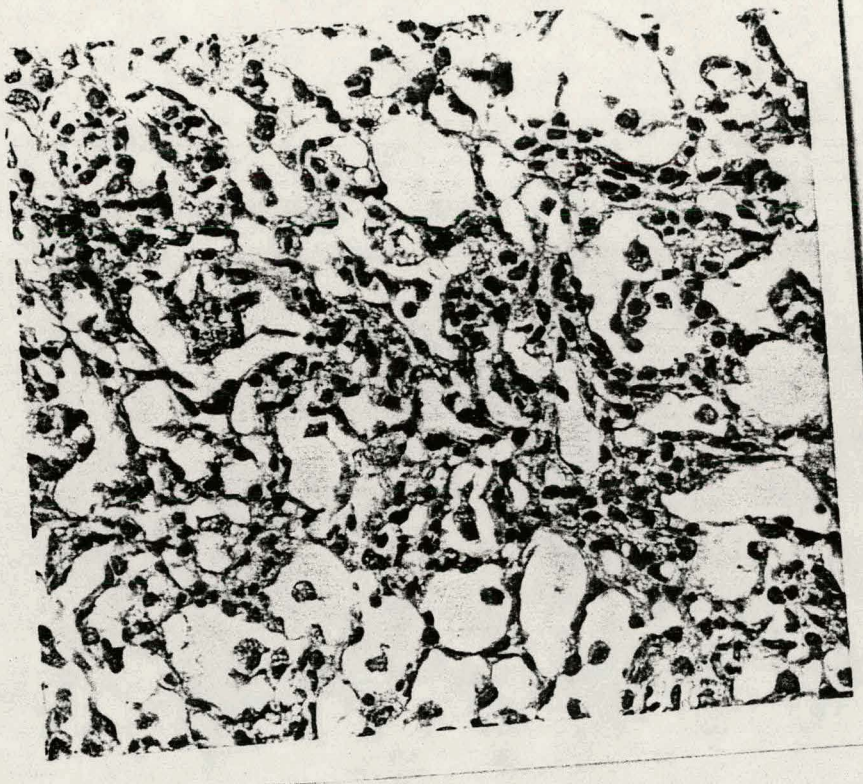

fig 4

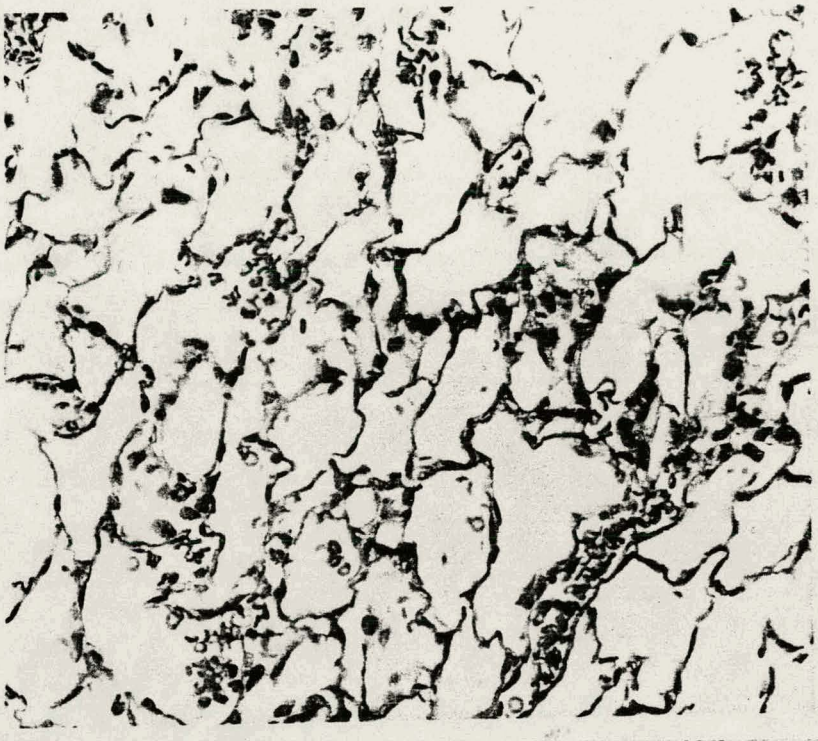

fig 5