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Biochemical and Connective Tissue Changes in Cyclophosphamide-induced Lung Fibrosis in Rats

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ABSTRACT. The present investigation was designed to characterize the biochemical and connective tissue components and to correlate the significance of morphological and biochemical perturbations in cyclophosphamide (CP)-induced lung fibrosis in rats. Lung fibrosis was induced in male Wistar rats by intraperitoneal injection of 20 mg/100 g body weight of CP, and their pneumotoxic derangements were characterized during an early destructive phase followed by a proliferative and synthetic phase. Serum angiotensin-converting enzyme (ACE) activity was higher in CP-treated rats at days 2, 3, 5, 7, and 11, but there was a significant decrease in lung ACE activity during the same time period. Elevated levels of β-glucuronidase activity were observed in the lung lavage fluid of CP-administered rats days 2, 3, 5, and 7. Lung myeloperoxidase activity was higher in CP rats. Of significance was the presence of collagenase and collagenolytic cathepsin in the lavage fluid of CP rats, when compared with the barely detectable levels in controls. A similar increase in these enzyme activities was also noticed in the lung tissue of CP rats during the same experimental period. Lavage fluid hydroxyproline content was higher in CP rats when compared with controls. Similarly, lung protein and DNA levels were elevated significantly after treatment with CP. The pulmonary histamine and serotonin contents were significantly higher in CP rats. The incorporation of [^{3}H]thymidine into lung total DNA, [^{3}H]proline into lung hydroxyproline, and [^{35}S]sulphate into lung glycosaminoglycan, measured as indicators of lung DNA, collagen, and glycosaminoglycan synthesis, respectively, was also higher in CP groups. Increased levels of hydroxyproline, elastin, hexosamine, total hexose, fucose, sialic acid, and uronic acid in the lungs of rats 14, 28, and 42 days after CP insult were characterized as biomarkers of CP-induced interstitial changes. These findings indicate that CP-induced lung fibrosis results in alterations not only in collagen synthesis and accumulation, but also in glycosaminoglycan and glycoprotein content. BIOCHEM PHARMACOL **56**;7:895–904, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. cyclophosphamide; lung fibrosis; collagen; collagenase; histamine; serotonin

CP§, a chemotherapeutic agent commonly used in the treatment of lymphomas, leukemias, and many solid tumors, produces diffuse interstitial and alveolar edema and inflammatory changes that progress to lung fibrosis in both animals and humans [1–6]. The major histologic abnormalities include endothelial cell damage, pneumocyte injury and necrosis, and fibroblast proliferation with fibrosis [1, 3, 7]. Although a great deal of evidence has been derived from clinical and histological studies in understanding the pathogenesis of CP-induced lung fibrosis, very little is known about the precise biochemical changes that drive the early destructive phase, which is followed by a proliferative and synthetic phase during the development of CP-induced lung fibrosis. In addition, detailed biochemical studies have not been carried out thus far to verify that interstitial components other than collagen increase [6] in CP-induced lung fibrosis. This is important because it suggests that this is a disease that involves not only collagen, but also the abnormal deposition and accumulation of many other extracellular matrix components as well [8]. In our earlier study, we characterized CP-induced early biochemical markers in lung lavage fluid and lavaged cells [9]. In the present study, we attempted to gain a better understanding of the biochemical changes seen upon intraperitoneal exposure of rats to CP and to correlate the reported histological evidence with connective tissue components and other biochemical markers associated with the development of CP-induced lung fibrosis.

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§ *Abbreviations:* ACE, angiotensin-converting enzyme; BALF, bronchoalveolar lavage fluid; CP, cyclophosphamide; GAG, glycosaminoglycan; and HHL, hippuryl-L-histidyl-L-leucine.

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MATERIALS AND METHODS

Materials

Acetylacetone, bacterial collagenase, bovine Achilles tendon collagen, BSA, calf thymus DNA, carbazole, chloramine-T, *p*-dimethylaminobenzaldehyde, *O*-dianisidine hydrochloride, glucuronolactone, hexadecyltrimethylammo-

nium bromide, hydrogen peroxide, L-hydroxyproline, *p*-nitrophenyl-β-D-*N*-acetylglucosaminide, thiobarbituric acid, and Triton X-100 were purchased from the Sigma Chemical Co. Cyclophosphamide (Endoxan Asta) was obtained from Khandelwal Laboratories Ltd. Radiochemicals ([^{3}H]thymidine, [^{3}H]proline, and [^{35}S]sulphate) were from the Bhabha Atomic Research Centre. All other chemicals were of analytical grade and commercially available.

Induction of Lung Fibrosis

Age- and weight-matched healthy, male Wistar rats weighing 100–125 g were housed five per cage with a photoperiod of 12 hr light/12 hr dark and given food and water *ad lib*. Rats were administered CP (20 mg/100 g body weight, i.p.) in 0.3 mL of sterile, triple-distilled water to induce lung fibrosis. Control rats received water alone. At various times thereafter (2, 3, 5, 7, 11, 14, 28, and 42 days postinjection), the animals were decapitated, blood samples were obtained and allowed to clot, and the serum was separated. The lungs were excised and chilled in ice-cold physiological saline to remove contaminating blood and adherent tissues, and they were stored at −70° until assayed.

Collection of BALF

At 2, 3, 5, 7, 11, 14, 28, and 42 days after CP exposure, six animals/treatment group were euthanized (sodium pentobarbital, 60–70 mg/kg body weight, i.p.) and the thoracic viscera was exposed. The trachea was cannulated, and the lung was lavaged with cold sterile physiological saline 5 times at a volume of 3 mL/wash cycle. The lavage fluid from each rat was centrifuged at 300 *g* for 10 min at 4° to sediment the cells prior to assay for biochemical markers. The entire operation was done in a cold room at 4°.

Lung DNA Synthesis

Lung DNA synthesis was assessed by measuring the incorporation of [^{3}H]thymidine into lung total DNA according to the method described by Witschi and Saheb [10]. Rats exposed to either water or CP were given 2 μCi/100 g body weight of [^{3}H]thymidine on days 2, 3, 5, 7, 11, 21, and 42 and killed after 90 min for the determination of specific activity of lung DNA. The results were expressed as cpm/μg of DNA.

Collagen Biosynthesis

Collagen synthesis was assessed following the method of Ramos *et al.* [11]. Lung slices were incubated in 5 mL of Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 50 μg/mL of ascorbic acid, 75 μg/mL of ferrous sulphate, 200 U/mL of penicillin, 200 μg/mL of streptomycin, and 30 μCi of [^{3}H]proline. Slices were equilibrated with 95%O_2/5%CO_2 and incubated for 4 hr at 37° in a controlled environment incubator shaker (New Brunswick Co., Inc.). At the end of the incubation period, lung samples were homogenized with a Polytron homogenizer in 10% (w/v) trichloroacetic acid and washed four times with 5% trichloroacetic acid. The trichloroacetic acid soluble fraction was hydrolyzed with 6 N hydrochloric acid (final concentration), and the hydroxyproline content and radioactivity of hydroxyproline were determined [12]. The results were expressed as cpm [^{3}H]hydroxyproline/hr/lung.

GAG Synthesis

Lung GAG synthesis was assayed by the incorporation of [^{35}S]sulphate ($Na_2{}^{35}SO_4$) into lung total sulphated GAGs. Injection of radioactive sulphate (50 μCi/100 g body weight, i.p.) was given 24 hr prior to killing the rats on days 7, 14, 21, 28, and 42. Labeled tissues were dried, delipidated, and analyzed for radioactive sulphate incorporation [13]. Corrections were made for background and decay. Total incorporation of label into GAG was expressed as cpm/mg dry tissue.

Biochemical Assays

Total protein was determined, by the method of Lowry *et al.* [14], using bovine serum albumin as the standard. Lung tissue DNA content was measured by the method of Burton [15], using calf thymus DNA as the standard. ACE activity of serum and lung homogenate was estimated according to the method of Cushman and Cheung [16] using HHL as the substrate. Lung tissue histamine content was determined according to the method of Shore [17] after butanol extraction and heptane purification. Lung tissue serotonin (5-hydroxytryptamine) content was quantitated according to the method described by Hanbrich and Denzer [18]. Myeloperoxidase activity of the lung tissue was determined by the method outlined by Bradley *et al.* [19].

Assay for Extracellular Matrix-degrading Enzymes

Collagenase activity in the bronchoalveolar lavage fluid was measured following the method of Christner *et al.* [20]. Collagenase activity in the lung tissue was estimated by the method of Woessner [21]. The activity of collagenolytic cathepsin in the tissue homogenate and lung lavage fluid was determined by the modified method of Anderson [22], using bovine tendon collagen as the substrate. The activities of β-glucuronidase and *N*-acetyl-β-D-glucosaminidase were assayed following the procedures of Kawai and Anno [23] and Moore and Morris [24], respectively.

Connective Tissue Macromolecules

The total collagen content of lung samples was determined by measuring hydroxyproline [25]. Ten milligrams of lung tissue was hydrolyzed with 6 N hydrochloric acid for 20 hr

TABLE 1. Body weight, lung weight, lung weight/body weight ratio, and levels of protein, DNA, histamine and serotonin in CP-treated rats

Treatment time (days)	Body weight (g)	Lung wet weight (g)	Lung weight/body weight ratio	Protein (mg/lung)	DNA (μg/lung)	Histamine (μg/g wet tissue)	Serotonin (μg/g wet tissue)
Control							
7	104 ± 18	0.68 ± 0.009	0.65 ± 0.02	75.7 ± 3.1	9.46 ± 0.5	3.6 ± 0.9	0.63 ± 0.1
14	113 ± 14	0.69 ± 0.009	0.61 ± 0.02	77.6 ± 2.8	9.29 ± 0.4	4.0 ± 1.1	0.59 ± 0.24
21	120 ± 20	0.70 ± 0.007	0.58 ± 0.03	76.5 ± 6.3	9.75 ± 0.4	3.8 ± 0.6	0.64 ± 0.19
28	126 ± 14	0.71 ± 0.007	0.56 ± 0.03	77.4 ± 5.5	10.2 ± 0.2	3.8 ± 1.0	0.67 ± 0.09
42	144 ± 17	0.72 ± 0.017	0.50 ± 0.02	81.4 ± 7.3	10.4 ± 0.3	3.9 ± 0.8	0.60 ± 0.18
CP							
7	90 ± 12	0.73 ± 0.030*	0.81 ± 0.05*	82.6 ± 7.6	12.5 ± 1.4*	6.9 ± 1.0*	0.86 ± 0.12†
14	86 ± 10‡	0.98 ± 0.013§	1.14 ± 0.05§	96.7 ± 6.4*	13.8 ± 0.5§	8.3 ± 1.8*	0.98 ± 0.19*
21	80 ± 11‡	1.15 ± 0.024§	1.44 ± 0.12§	110.7 ± 3.8§	13.0 ± 0.8*	10.6 ± 2.1*	1.69 ± 0.25*
28	76 ± 15‡	1.38 ± 0.042§	1.81 ± 0.10§	117.0 ± 11.7*	14.5 ± 1.1*	13.6 ± 2.2*	2.14 ± 0.76§
42	66 ± 13‡	1.53 ± 0.058§	2.32 ± 0.07§	144.1 ± 7.2§	15.5 ± 0.9§	16.8 ± 1.5§	3.78 ± 1.5§

Values are means ± SD of six observations.
†Significantly ($P < 0.01$, †$P < 0.05$) higher than controls.
‡Significantly ($P < 0.01$) lower than controls.
§Significantly ($P < 0.001$) higher than controls.

at 110°, evaporated to dryness, resuspended in triple-distilled water, and the hydroxyproline content estimated. The collagen content was calculated by multiplying the hydroxyproline value by the factor 7.46. The collagen content was expressed as μg of hydroxyproline/mg of dry tissue.

The elastin content of lung samples was determined by the method of Neuman and Logan [26]. A known amount of lyophilized lung sample was autoclaved for 3 hr at 15 p.s.i. (121°). The autoclaving was repeated twice with intermittent washing of the sample. After final autoclaving, the residue left behind was washed thoroughly with distilled water and then was hydrolyzed for 20 hr with 6 N hydrochloric acid at 110°. The hydroxyproline content was then determined from the hydrolysate after evaporating hydrochloric acid. The percentage of elastin in rat lung was calculated from the expression (μg of hydroxyproline/μg of sample) × factor × 100. The factor for rat is 43.4 [26].

Lung tissue uronic acid [27] content was determined in dry, defatted [successive extraction at 60° with ethanol: ether, 3:1 (v/v) followed by chloroform:methanol, 1:1 (v/v)] and papain digested tissue.

Determination of BALF Hydroxyproline

Aliquots of cell-free lung lavage fluid were hydrolyzed with 6 N hydrochloric acid for 20 hr at 110°, evaporated to dryness, and resuspended in triple-distilled water, and the hydroxyproline content was estimated [25].

Extraction and Determination of Lung Glycoproteins

The lungs were dissected, rinsed in ice-cold physiological saline to remove blood and contaminating tissues, weighed, and finely minced with scissors. Tissues were homogenized in 0.15 M sodium chloride using a Polytron homogenizer at 4°.

Lung tissue hexosamine levels were measured following the procedure adopted by Boas [28]. Fucose content in the tissue homogenate was determined utilizing the method described by Dische and Shettles [29]. The content of sialic acid in tissue samples was estimated using thiobarbituric. acid as described by Warren [30]. Total hexose was determined by the method of Dubois *et al.* [31] using phenol as the specific organic colour developing reagent.

Statistical Analysis

Data were calculated as means ± SD of six observations, and comparison between groups was done with Student's *t*-test.

RESULTS

General Characteristics of CP-treated Rats

At a dose of 20 mg/100 g body weight, CP caused a mortality rate of 25% that occurred between weeks 1 and 2 of injection. Gross examination of rats showed alopecia in more than 60% of rats treated with CP. CP injection was also associated with ocular toxicity (50%). Haematuria was prominent in CP-treated rats. No morphologic change was observed in control rats. The lungs of the control rats were macroscopically normal.

Body Weight and Lung Weight

The body weights of the vehicle-treated and CP-treated rats were recorded every week. All vehicle-treated control rats continued to gain weight throughout the study (Table 1). However, CP was found to impair body weight gain. The body weights of the CP-treated rats had declined and were significantly ($P < 0.01$) less than controls at days 14, 21, 28, and 42. Lung wet weight was elevated significantly

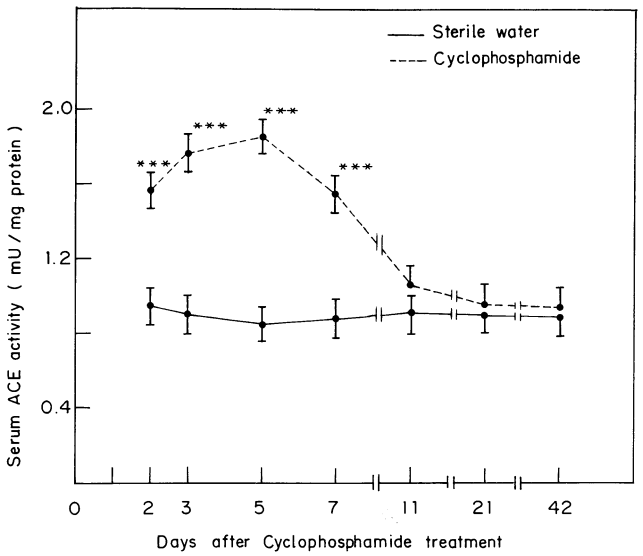

FIG. 1. Effect of CP administration on serum ACE activity. Values are means ± SD of six observations. Key: (***) significantly ($P < 0.001$) higher than control rats.

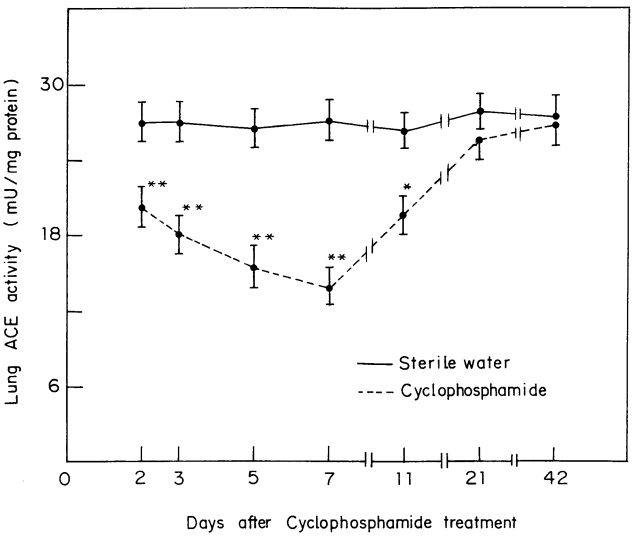

FIG. 2. Lung ACE activity in normal and fibrotic rats. Values are means ± SD of six observations: Key: (*) and (**) significantly ($P < 0.05$ and $P < 0.01$, respectively) lower than control rats.

($P < 0.01$) in CP-injected rats as compared with that of controls at all times examined (Table 1). In a similar vein, the lung weight/body weight ratio of CP rats was significantly ($P < 0.01$) higher as early as 7 days and continued to increase progressively thereafter (Table 1).

ACE Activity

Serum ACE levels in CP-treated rats increased significantly ($P < 0.001$) at 2, 3, 5, and 7 days (Fig. 1), but thereafter fell to control levels by day 42. On the contrary, lung ACE levels were significantly lower in CP-treated rats at 2, 3, 5, and 7 days ($P < 0.01$) and also at 11 days ($P < 0.05$) (Fig. 2), but returned to control levels by day 42.

Myeloperoxidase Activity

Lung myeloperoxidase activity, expressed in terms of units per lung, increased significantly ($P < 0.01$) as compared with controls at days 2, 3, 5, 7, 11, and 21 following CP injection (Fig. 3). The maximum increase in the enzyme activity was noticed at day 7; however, this activity fell to control levels at day 42.

BALF Collagenolytic and Glycohydrolase Activities

Several enzymes are involved in the destruction of lung connective tissue, and one or more of these enzymes might be released during pulmonary inflammation. To determine if these enzymes are involved in the breakdown of connective tissue components during the early stage of fibrosis, we measured both the collagenolytic enzymes and glycohydrolases in CP-treated rats. Time-dependent increases in the activities of collagenase (Fig. 4), collagenolytic cathepsin (Fig. 5), and β-glucuronidase (Fig. 6) in lung lavage fluid 2, 3, 5, and 7 days after CP exposure were observed. This response was decreased by day 42. However, no lavage fluid collagenolytic activities were found in control rats.

Lavage Fluid Hydroxyproline

There was a significant increase in the lavage fluid hydroxyproline content in CP rats (Fig. 7) on days 3 ($P < 0.01$), 5, 7 ($P < 0.001$), and 11 ($P < 0.01$) when compared with controls; however, it returned to normal values by day 42.

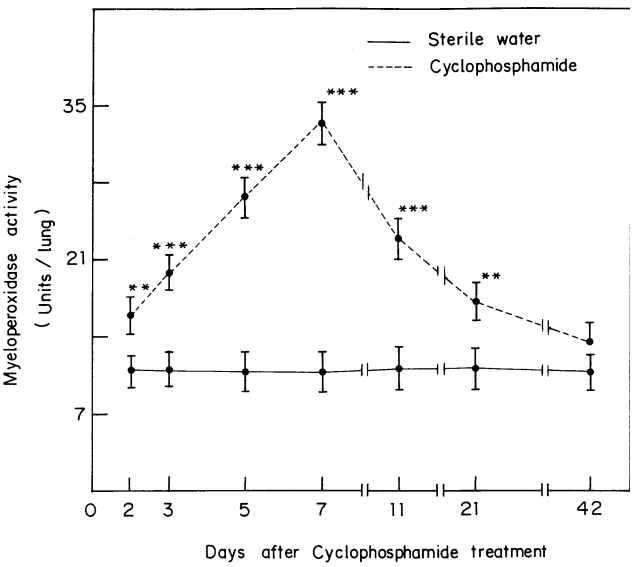

FIG. 3. Sequential changes in lung myeloperoxidase activity in rats exposed to CP. Data are means ± SD of six observations. Key: (**) and (***) significantly ($P < 0.01$ and $P < 0.001$, respectively) higher than control rats.

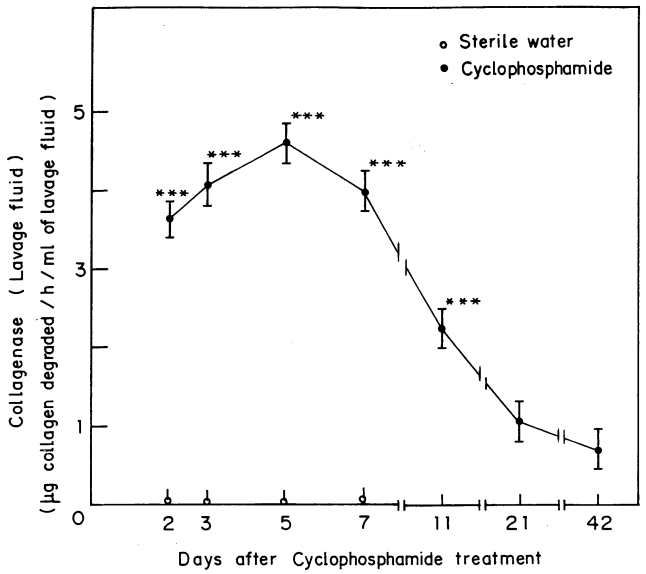

FIG. 4. Sequential changes in the lavage fluid collagenase activity in rats exposed to CP. Data are means ± SD of six observations. Key: (***) significantly ($P < 0.001$) higher than control rats.

Lung DNA Synthesis

The increase in DNA content of lungs of CP-exposed rats was accompanied by an increase in the incorporation of radioactive thymidine into total lung DNA (Fig. 8). The increase in [^{3}H]thymidine incorporation into DNA occurred on days 3 and 5, peaked on days 7 and 11, but thereafter fell to control values by day 42.

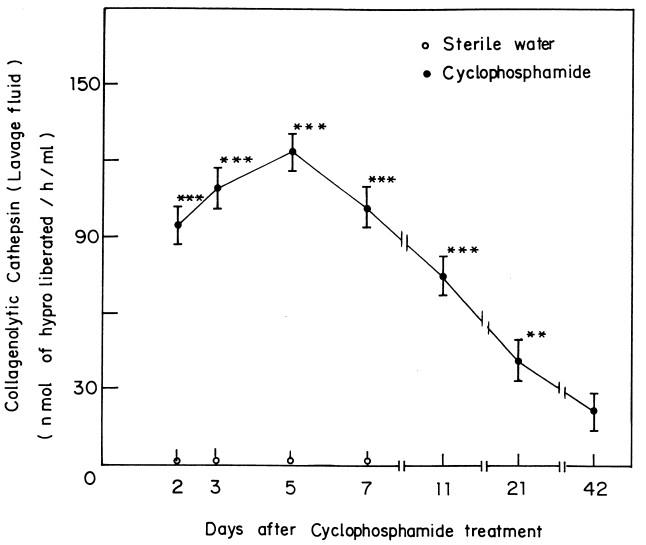

FIG. 5. Time–course alterations in the lavage fluid collagenolytic cathepsin activity in rats exposed to CP. Hypro-hydroxyproline. Values are means ± SD of six observations. Key: (**) and (***) significantly ($P < 0.01$ and $P < 0.001$, respectively) higher than control rats.

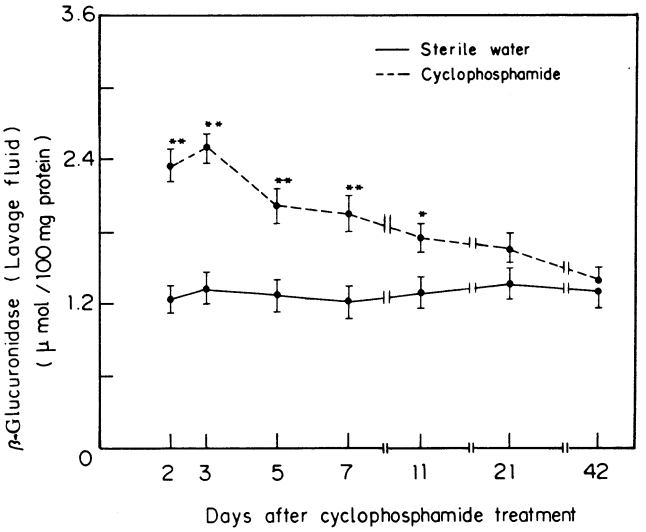

FIG. 6. CP-induced alterations in lavage fluid β-glucuronidase activity in rats. Data are means ± SD of six observations. Key: (*) and (**) significantly ($P < 0.05$ and $P < 0.01$, respectively) higher than control rats.

Lung Collagen Synthesis

Collagen biosynthesis, measured by the formation of [^{3}H]hydroxyproline in the lung tissue of rats exposed to CP, is shown in Fig. 9. Fourteen days after CP injection, the rate of hydroxyproline synthesis was elevated. The elevation in lung hydroxyproline synthesis was also observed 21, 28, and 42 days after CP exposure. However, the same treatment did not result in any significant change in hydroxyproline synthesis in the lungs at day 7.

Lung GAG Synthesis

The incorporation of [^{35}S]sulphate into lung GAG is presented in Fig. 10. Following CP injection, there was a

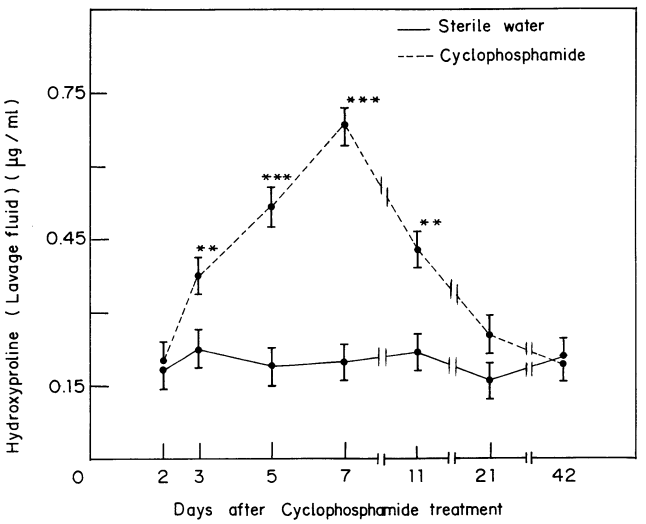

FIG. 7. Effect of CP administration on lavage fluid hydroxyproline content. Values are means ± SD of six observations. Key: (**) and (***) significantly ($P < 0.01$ and $P < 0.001$, respectively) higher than control rats.

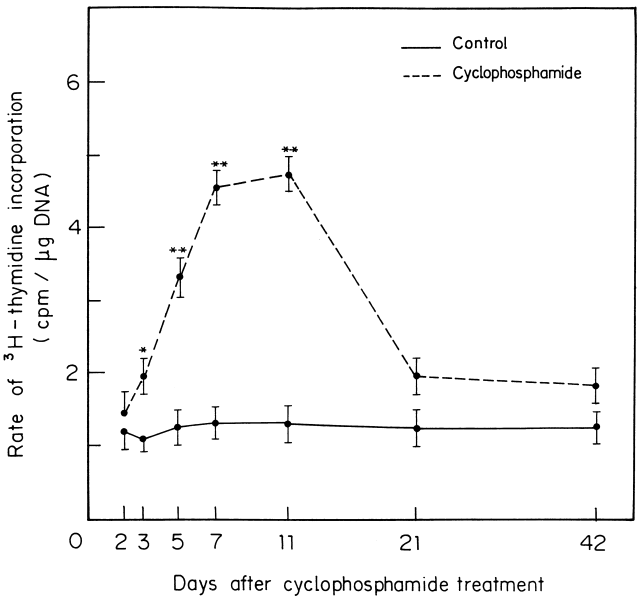

FIG. 8. Rate of [^{3}H]thymidine incorporation into lung DNA at various times after CP administration. Values are means ± SD of six observations. Key: (*) and (**) significantly ($P < 0.05$ and $P < 0.01$, respectively) higher than control rats.

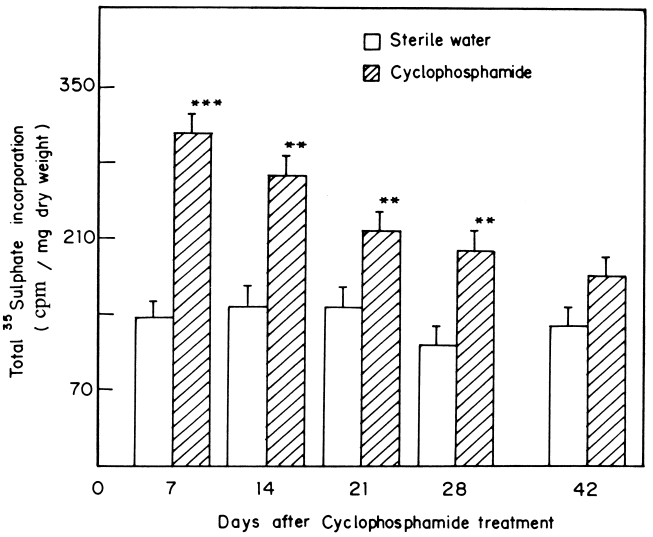

FIG. 10. Rate of [^{35}S]sulphate incorporation into lung GAG at various times after CP treatment. Values are means ± SD of six experiments. Key: (**) and (***) significantly ($P < 0.01$ and $P < 0.001$, respectively) higher than control rats.

significant ($P < 0.01$) increase in the total incorporation of [^{35}S]sulphate into lung GAG when compared with that of controls, The maximum [^{35}S]sulphate incorporation occurred 7 days after CP and then gradually declined by day 42, but still was markedly higher than controls.

Lung Biochemical Components

Table 1 shows the alterations in biochemical constituents of lungs of CP-treated rats. Lung total protein content was increased significantly in CP-injected rats when compared with controls. The DNA content was also found to be increased at all time points examined. Levels of histamine and serotonin were measured in lungs of control and CP-exposed rats. The data presented indicate that these biogenic amine contents in lungs of CP-treated rats were increased above control values at 7, 14, 21, 28, and 42 days after CP treatment.

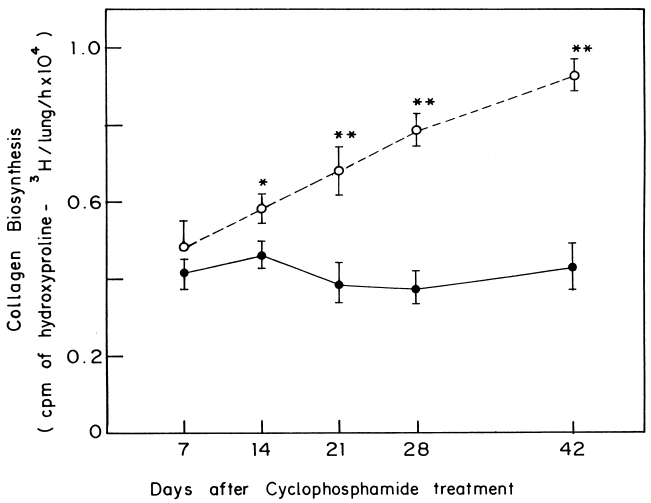

FIG. 9. Rate of [^{3}H]proline incorporation into lung collagen and changes in the rates of collagen synthesis in lung explant cultures at various times after CP treatment. Values are means ± SD of six observations. Key: (*) and (**) significantly ($P < 0.05$ and $P < 0.01$, respectively) higher than control rats.

Lung Extracellular Matrix Components

Administration of CP to rats caused a progressive rise in lung connective tissue components (Table 2). The hydroxyproline content of lungs in CP-administered rats was significantly higher than in control rats at 14, 21, 28, and 42 days after CP administration. Lung elastin content in CP-treated rats was also significantly higher than in control groups at 7, 14, 21, 28, and 42 days. The present study also demonstrated that CP treatment resulted in comparable increases of lung hexosamine, total hexose, fucose, sialic acid, and uronic acid content over the same experimental period.

Lung Collagenolytic and Glycosidase Activities

Table 3 illustrates the results of CP-induced changes in the matrix degrading enzymes. The results indicate that the administration of CP resulted in a significant increase in lung levels of collagenase, collagenolytic cathepsin, *N*-acetyl-β-D-glucosaminidase, and β-glucuronidase on days 2, 3, 5, and 7. The results also demonstrate that these values were reduced significantly ($P < 0.01$) by day 42 when compared with control rats.

TABLE 2. Levels of hydroxyproline, elastin, hexosamine, total hexose, fucose, sialic acid and uronic acid in CP-treated rats

Treatment time (days)	Hydroxyproline (μg/mg dry tissue)	Elastin (mg/g dry tissue)	Hexosamine (mg/g protein)	Total hexose (mg/g protein)	Fucose (mg/g protein)	Sialic acid (mg/g protein)	Uronic acid (mg/g dry tissue)
Control							
7	16.6 ± 0.5	18.2 ± 2.1	37.5 ± 2.5	52.3 ± 7.8	17.7 ± 3.5	26.1 ± 4.6	2.24 ± 0.24
14	17.5 ± 0.9	16.0 ± 1.9	35.1 ± 2.3	48.5 ± 7.5	16.7 ± 3.7	29.3 ± 6.5	2.30 ± 0.33
21	15.9 ± 0.9	18.2 ± 2.5	37.8 ± 1.6	51.2 ± 7.3	17.6 ± 2.4	29.8 ± 7.4	2.21 ± 0.24
28	17.3 ± 0.9	17.6 ± 1.0	37.5 ± 2.3	52.8 ± 11.6	16.4 ± 4.0	27.4 ± 5.1	2.29 ± 0.38
42	18.1 ± 0.3	18.4 ± 2.1	37.7 ± 1.3	55.6 ± 4.9	18.1 ± 2.4	25.3 ± 3.7	2.34 ± 0.42
CP							
7	13.6 ± 2.6	21.2 ± 1.4*	46.1 ± 1.3†	85.0 ± 6.0†	29.1 ± 4.5†	42.5 ± 10.1†	2.80 ± 0.31†
14	21.8 ± 1.5†	24.1 ± 1.6†	49.2 ± 1.6‡	79.9 ± 5.3†	31.4 ± 6.1†	41.7 ± 7.9*	2.94 ± 0.42*
21	24.2 ± 1.8†	28.0 ± 2.3†	52.4 ± 2.6‡	74.8 ± 5.1†	43.0 ± 11.1†	38.3 ± 8.4*	3.09 ± 0.36†
28	29.9 ± 0.9‡	29.2 ± 1.9‡	58.5 ± 4.4‡	73.5 ± 6.0†	32.0 ± 3.5†	36.5 ± 4.7†	3.14 ± 0.45†
42	32.7 ± 2.5‡	32.1 ± 2.6†	59.1 ± 2.9‡	72.9 ± 7.9†	31.3 ± 8.8†	38.6 ± 9.5†	3.50 ± 0.51†

Values are means ± SD of six observations.
*–‡Significantly ($*P < 0.05$, $†P < 0.01$, $‡P < 0.001$) higher than controls.

DISCUSSION

The administration of CP caused a decrease in body weight coupled with a significant increase in lung wet weight. The body weight changes may be due to cytotoxic and wasting disease [32], and the increase in lung wet weight may be due to the result of interstitial fluid accumulation, edema, increased synthesis of protein, DNA, and accumulation of extracellular matrix components. In addition, hyperplasia could also contribute to increased lung weight. As a further indication of cellular damage, increased serum ACE levels and decreased levels of the same in lungs of CP-treated rats were seen. It has been suggested that one of the early events that precede fibrosis is damage to the lung capillary endothelium, and several investigators have monitored serum ACE levels as a marker for early lung damage [33, 34]. The data presented here support the view that changes in the activities of this enzyme correlate well with the early events that precede fibrosis in CP-treated rats [9].

A number of studies have utilized the [^{3}H]thymidine incorporation into lung DNA in assessing the type II alveolar epithelial cell proliferation that arises after chemical or drug-induced lung damage [5, 10, 35, 36]. Results of the present study indicate that increased incorporation of thymidine occurred on days 3 and 5, peaked on days 7 and 11, and returned to control levels on day 42. These thymidine incorporation findings suggest proliferation of type II alveolar epithelial cells after CP treatment. Our results are in good agreement with the findings of Witschi *et al.* [37], who have demonstrated peak alveolar cell proliferation 5 days after CP injection in mice. While it is likely that type II pneumocytes are responsible, at least in part, for newly synthesized DNA, a wide variety of other

TABLE 3. Sequential changes in matrix degrading enzymes in rat lungs after treatment with CP

Treatment time (days)	Collagenase*	Collagenolytic cathespin†	N-Acetyl-β-D glucosaminidase‡	β-Glucuronidase‡
Control				
2	24.1 ± 3.2	330 ± 26.6	44 ± 7.7	31 ± 3.1
3	20.8 ± 4.1	306 ± 16.9	43 ± 5.4	33 ± 2.8
5	22.0 ± 2.1	315 ± 17.0	39 ± 3.9	35 ± 1.9
7	23.2 ± 2.6	320 ± 19.3	40 ± 4.4	30 ± 2.3
42	23.5 ± 1.9	315 ± 15.5	41 ± 3.3	33 ± 1.1
CP				
2	29.0 ± 4.1§	397 ± 21.9§	52 ± 5.9§	38 ± 2.4§
3	33.6 ± 3.6‖	420 ± 25.1‖	57 ± 6.3‖	44 ± 4.6‖
5	38.3 ± 3.8‖	485 ± 18.9‖	61 ± 5.8‖	49 ± 3.9‖
7	33.2 ± 3.9‖	526 ± 16.8¶	65 ± 4.4‖	52 ± 1.8¶
42	16.8 ± 3.1**	263 ± 14.4**	30 ± 4.2**	24 ± 2.0**

Data are means ± SD of six experiments.
*Expressed in μg of collagen degraded/mg of collagen.
†Expressed in nmol hydroxyproline liberated/mg of protein.
‡Expressed in μmol *p*-nitrophenol liberated/hr/100 mg of protein.
§–¶Significantly ($§P < 0.05$, $‖P < 0.01$, $¶P < 0.001$) higher than controls.
**Significantly ($P < 0.01$) lower than control groups.

cell types may also contribute to the response as well [38]. In addition to corroborating the type II cell proliferation to DNA synthesis, data from our own studies [9] indicate increased levels of alkaline phosphatase and acid phosphatase activities in the lung lavage fluid of CP-injected rats over the same time period. These alterations, therefore, confirm an early type I pneumocyte injury followed by hypertrophy and hyperplasia of type II pneumocytes [1, 7]. Since total lung DNA was elevated, there appeared to be increased cellularity in the damaged lung.

Early infiltration of neutrophils, eosinophils, and macrophages is widely recognized as a frequent response in the injured alveolar tissue in experimental animals exposed to lung toxicants [39, 40] and in various fibrotic lung disorders [41, 42]. Accumulation of neutrophils is a prominent feature of the acute phase of lung injury caused by CP [1]. These inflammatory lymphocytes, neutrophils, and macrophages have been demonstrated to be implicated in the destruction of connective tissue by the release of proteolytic enzymes and the generation of free radicals [43–45]. Free radical production in these conditions is clearly linked to cellular injury, membrane perturbation with increased vascular permeability resulting in a leakage of enzyme. A similar release of enzymes and free radical generation from damaged alveolar tissue occurred after CP exposure [9]. We found that CP intoxication produced a significant increase in *N*-acetyl-β-D-glucosaminidase [9] and β-glucuronidase activities in the lung and lavage fluid on days 2, 3, 5, and 7. Myeloperoxidase (an index of neutrophil content) converts Cl^- to hypochlorous acid, which is known for its biocidal property in oxidizing a variety of vital cellular components [46]. Thus, the ability to estimate the activity of myeloperoxidase in the lung provides a valuable diagnostic index of the intensity of inflammation. The lung myeloperoxidase activity in the CP group was elevated significantly as compared with that of controls.

In the early phase of CP lung injury, there is a transient increase in the tissue levels of collagenase and collagenolytic cathepsin activities. Interestingly, the increased levels of collagenase and collagenolytic cathepsin activities in the lung tissue coincided with the levels of these enzymes in lung lavage fluid. Its appearance in lung and lavage fluid has been described in idiopathic pulmonary fibrosis [47], an experimental model of lung fibrosis [11], and patients with adult respiratory distress syndrome [20]. The major finding of this study was the evidence for degradation of lung connective tissue during the acute inflammatory stage. Degradation of lung connective tissue was also demonstrated by increased levels of hydroxyproline in lung lavage fluid of CP-treated rats. It is of interest to note that the peak time of increased hydroxyproline in lavage fluid corresponded to the time of peak lavage albumin content [9] and lung myeloperoxidase activity, suggesting that degradation was maximal at the time of intense inflammatory processes. It is suggested that the hydroxyproline-containing material in lavage fluid represents collagen fragments released from damaged lung parenchyma [48]. These *in vivo* findings imply matrix degradation at sites of alveolar inflammation that contribute to matrix reorganization. The data presented here suggest that the early degradation of collagen in the lungs of CP-treated rats is a feature of the injurious response, and that the repair process involves the synthesis of excessive connective tissue components. In addition to establishing the early degradation of collagen, our results also revealed a decrease in collagenolysis and collagenolytic activities in the latter stages of fibrosis, raising the possibility that a decrease in collagen degradation and an increase in collagen biosynthesis may be key features in the maintenance and progression of fibrosis. Thus, the results of our studies were comparable to those reported in silica-treated rats [11].

Histamine and serotonin, the vasoactive chemotactic mediators released from mast cells sequestered in the lung during inflammatory conditions, have been reported as putative mediators in the chain of events leading to fibrosis [49, 50]. Mast cell hyperplasia has been demonstrated in patients with fibrotic lung disorders [51], in scleroderma [52], and in experimental animals exposed to asbestos [53], silica [54], and bleomycin [55]. In the present study, we found that both histamine and serotonin were elevated in the lung tissue of CP rats between 7 and 42 days of treatment. Although we did not actually document mastocytosis either histologically or by mast cell counting, we were able to document biochemically increased levels of biogenic amines during the development of fibrosis that strongly correlated with other biomarkers of pulmonary fibrosis.

Several lines of evidence indicate histologically and ultrastructurally that an early destructive phase followed by a proliferative and synthetic phase occurs in experimental animals following exposure to pulmonary toxicants [56–59]. Biochemical examination of the lungs of CP-treated rats indicates increased levels of hydroxyproline, elastin, hexosamine, total hexose, fucose, sialic acid, and uronic acid. The observed increases in glycoprotein moieties may be interpreted on the basis of elevated levels of the glycoprotein-synthesizing enzymes in the inflamed state and suggest that they are sequelae of an inflammatory process during the development of fibrosis [60]. Evidence has also been presented that pulmonary elastin and hexosamine contents are increased in bleomycin-injured lung tissue [61]. The marked increase in connective tissue components at 7, 14, 21, 28, and 42 days after CP exposure suggests a rapid increase in the rate of connective tissue synthesis. We believe that the increase in the extracellular matrix components of the lung tissue could be associated with the activation of fibroblasts to divide, proliferate, and synthesize collagen and other matrix constituents, a process mediated by a host of substances that include immune complexes, serum components, growth factors, and cytokines, following tissue injury and during host inflammatory response [62, 63]. Indeed, evidence of increased incorporation of [^{3}H]proline into lung collagen and [^{35}S]sulphate into lung glycosaminoglycans 7, 14, 28, and 42 days after

CP administration would suggest that CP may alter the growth and metabolic activity of fibroblasts.

We conclude that CP-induced lung fibrosis is characterized by initial destructive damage followed by a reparative phase dominated by excessive deposition of collagenous and noncollagenous proteins.

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