Correlation of Biochemical and Morphologic Manifestations of Acute Pulmonary Fibrosis in Rats Administered Paraquat*

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Rats were administered 24 mg/kg of paraquat intraperitoneally. One, two, three, six and seven days after this injection, the rats were evaluated by several toxicologic, biochemical, and morphologic criteria. These included determinations of weight loss, visible lung hemorrhage, pulmonary edema, in vitro protein and collagen biosynthesis rates of lung tissue, and staining properties of lung sections. Morphologic evidence of mild edema was seen at day 1, while more severe edema was observed at days 2 through 7. Weight loss was apparent from day 1 throughout the experiment. In vitro protein biosynthesis rates by tissue were elevated above control values from day 3 onwards, while collagen biosynthesis rates were elevated from day 2 onwards. Increased staining with the Gomori reagent was observed from day 1, while increased staining with van Gieson's or Masson's trichrome appeared by day 2 or 3.

Pulmonary fibrosis is a severe, debilitating, and often fatal human disease. Although several animal models have been used to study experimentally-induced pulmonary fibrosis, none of these models has proven totally satisfactory from the point of view of being technically simple, reproducible, and/or exactly mimicking the pathologic features of the human disease.

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We have recently reported biochemical studies using an animal model in which acute fibrosis is induced over several days by the intraperitoneal injection of the LD50 dose of paraquat into rats. Using a lung explant system based upon the methods of Crystal and co-workers, we have demonstrated that there is an increased rate of collagen biosynthesis by lung explants prepared from rats three days after the injection of paraquat. In the present paper, we present a correlative study of effects of paraquat on rat lungs at various times after injection of the LD50 dose. The time course of development of pulmonary fibrosis after paraquat administration is described, and is documented both microscopically by increased appearance of collagen and reticulin visualized histologically, and biochemically as an increased rate of collagen biosynthesis by lung explants.

Materials and Methods

Chronic respiratory disease-free male rats of the Sprague-Dawley strain were obtained from Hilltop, Scottsdale, Pennsylvania. Gramaxone (Chevron Chemical Co., Richmond, California) was used as the source of paraquat. All other chemicals and materials were of reagent grade or better, and were obtained as described elsewhere. Rats (300-500 gm) were injected intraperitoneally with a 2.9 percent paraquat solution (w/v, Gramoxone diluted with water), at a dose of 24 mg/kg. After the appropriate number of days, during which time the animals were fed water and chow ad libitum and allowed to breathe filtered air, randomly-chosen rats were weighed and overdosed with sodium pentobarbital. The chests were opened, and visual estimates were made of the percentage of lung surface that appeared hemorrhagic. The right cranial lobe of each rat was clamped with a hemostat, and the lung was perfused via the pulmonary artery with 0.15 M NaCl solution. The right apical lobe was then removed, dried with gauze, and weighed before and after drying for at least 48 hours at 110-120°C for determination of the wet-to-dry-weight ratio. The left lung lobe was tied off with silk thread, removed, and minced into pieces approximately 1 mm³ for in vitro incubation with radioactive proline. The three remaining lobes were removed with the heart en bloc, then perfused via a tracheal cannula with formol-saline solution under 30 cm of pressure at room temperature for 24 hours.

Sections of lung were then taken from each of the three fixed lobes approximately one-third of the distance apically from the entry point of the main bronchus. Sections were embedded in paraffin, sectioned at six micrometers, and appropriately stained.

Results

Gross Evaluations of Toxicity

Table 1 shows the sampling intervals, numbers of

Acute Pulmonary Fibrosis in Rats Given Paraquat

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Table 1—Effects of Paraquat on Rats at Various Times After Its Administration

<table>
<thead>
<tr>
<th>Days after Paraquat Injection</th>
<th>No. Rats Evaluated*</th>
<th>Rat Weight, Average % of Initial Weight**</th>
<th>% of Lungs Hemorrhagic, Average by Visual Estimate†</th>
<th>Wet/dry Weight Ratio of Right Apical Lobe, Mean ± SD‡</th>
<th>Masson’s Trichrome Stain§</th>
<th>Gomori’s Stain§</th>
<th>Hematoxylin and Eosin Stain¶</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2</td>
<td>90</td>
<td>0</td>
<td>4.68 ± 0.18</td>
<td>0</td>
<td>±</td>
<td>±</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>87</td>
<td>35</td>
<td>5.95 ± 0.29†</td>
<td>±</td>
<td>+</td>
<td>±</td>
</tr>
<tr>
<td>3</td>
<td>2</td>
<td>84</td>
<td>82</td>
<td>7.62 ± 0.55‡</td>
<td>±</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>1</td>
<td>74</td>
<td>80</td>
<td>5.74</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>3</td>
<td>76</td>
<td>88</td>
<td>6.03 ± 0.29‡</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

*Two control rats were evaluated in parallel each day.
**Percentage of initial weight ranged from 99 percent to 102 percent for the control group.
†All control lungs were scored as 0 percent hemorrhagic in this measurement.
‡Wet/dry weight ratio for control rats was 4.68 ± 0.28 (n = 9, one sample was lost).
§Differences significant (Student’s t-test) at the P < 0.01 level, assuming a normal distribution of the data.
¶Sections were evaluated on a scale of 0 = indistinguishable from normal; ± = marginal subjective increase; + = increased level of staining; ++ = very strong increase in staining.

Days 1 through 7, we saw the same types of changes that were evident at day 2, although the response was more severe.

Biochemical Measurements in Lung Explants

Paraquat administration increases the proline pool size of lung slices.12 We found no significant differences in the proline content of lung slices from control and paraquat-injected rats one or two days after paraquat administration. By day 3, the tissue content of proline had increased to 179 percent of the control value (289 ± 30 versus 518 ± 75 ng/50 μl tissue extract, mean ± SD), while at day 6, the increase was to 320 percent of the control value (345 ± 58 versus 1105 ± 153) and at day 7 the proline content was 329 percent of the control value (336 ± 71 versus 1103 ± 282). Since we add a constant amount of tritiated proline to each tissue incubation vial, the actual specific activity of the radioactive precursor is lower during the incubation period in the lung slices from rats that contain higher concentrations of endogenous (unlabelled) proline. Thus, comparisons of rates of biosynthesis of total protein and of collagen (hydroxyproline) in lungs from control and paraquat-treated rats must be corrected for the lower specific activity of proline in tissue from paraquat-treated rats. We therefore express rates of protein and collagen synthesis as nanograms of proline (total protein) or of hydroxyproline (collagen) synthesized per hour per mg of lung-slice protein.

The rates of total protein synthesis were calculated from the slope of plots of in vitro incorporation of [3H]proline into acid-precipitable material versus time over a three-hour period. As shown in Figure 1, the overall rates of protein synthesis by lungs from control and from paraquat-treated rats were similar at days 1 and 2. Subsequently, the tissue from exposed rats manifested a higher rate of protein synthesis, eventually maintaining a rate approximately four-fold higher than tissue from control animals.

We evaluated the rate of collagen biosynthesis by the lung slices, measured as the incorporation of [3H]proline into acid-insoluble [3H]hydroxyproline, a good marker for collagen in lungs.13 As shown in Figure 2, there is no discernible effect of paraquat administration on collagen synthesis rate at day 1. By the second day after paraquat injection, however, the rate of hydroxyproline synthesis was significantly elevated (P < 0.05), while on days 3 through 7 the hydroxyproline synthesis rate continued to increase to very high levels as compared with the control rats. The apparent difference in synthesis rate (determined by calculating the slopes of the graphs in Figure 2) between lung slices prepared from control and paraquat-treated rats is...
about eight-fold at day 3, about 16-fold at day 6, and about 13-fold at day 7. These values range from two to fourfold higher than the maximal increase in overall protein biosynthesis rate (Fig 1).

**Histopathologic Evaluations of Lung Changes**

We will describe only those changes observed that apparently relate to the correlative biochemical and gross pathologic observations described above. On day 1 (24 hours after paraquat administration) the most striking pathologic event noticed was mild, relatively acellular, perivascular edema. This finding was noted throughout all three lobes examined and affected all sizes of vessels. Occasional peribroncholar edema was noted. There was little alveolar edema and no alveolar cellular infiltration. Some capillary congestion was noted. Material staining with Gomori's silver stain was present, especially noticeable as thicker bundles of reticular fibers running longitudinally through the septae and as a slightly increased prominence of reticular wreaths surrounding the capillary enclosures. On day 2, occasional mononuclear cells were observed in the edematous areas, especially around the vessel walls. Little alveolar edema or interstitial edema was seen, and the alveolar architecture appeared intact. Focal areas of alveolar infiltration by macrophages and mononuclear cells were observed. The material staining with Gomori's was more prevalent and more densely stained than at day 1. Collagen, as visualized with van Gieson's or Masson's trichrome stains, first appeared to exceed the normal amounts two or three days after injection of paraquat, espe-
characteristic pathologic finding in pulmonary fibrosis, an apparent increased deposition of collagen in the lung parenchyma, is observed within three days of paraquat administration. Severe fibrosis is observed within six days after injection of this agent (Fig 3). Second, there is an obvious correlation between the various indicators of gross damage evaluated (Table 1), the biochemical findings in the tissue mince experiments (Fig 1 and 2), and the histopathologic findings (Table 1, Fig 3), as a function of time after administration of paraquat. Especially evident are the relationships between the temporal appearance of inflammatory edema and changes in the wet-to-dry-weight ratios, and between the increased collagen synthesis rate and the appearance of morphologic indicators of pulmonary fibrosis. While such a correlation does not prove a cause-and-effect relationship, it is certainly consistent with such being the case.

Rats appear to be more susceptible than humans to death from the early edematous phase of paraquat intoxication. Therefore, the etiology of paraquat-induced fibrosis in humans has been studied better, although it is stated\(^1\) that the pathogenesis of pulmonary fibrosis is the same in both species. Very little work with special collagen stains has been reported. Failure of areas of hemorrhage and adjoining intra-alveolar spaces filled with "proliferoblasts" (areas at a more advanced stage of paraquat intoxication than described in the present study) to stain with van Gieson’s stain has been reported.\(^1\) Such findings have led in part to there being a distinction made between paraquat-induced pulmonary fibrosis and the more common (in human disease states) fibrosing alveolitis. Other reports have stated that the alveolar matrix stains only faintly with van Gieson’s reagent and consists largely of "immature collagen" (undefined by the authors) and fibrin.\(^2\) In addition, there is evidence,\(^2,\) based on staining with elastic van Gieson’s, for perivascular and peribronchial occurrence of fine collagen fibers and reticulin. Vijeyaratnam and Corrin\(^1\) report using Gomori’s stain in a study of rats receiving high doses of paraquat (25 mg/kg); they report an apparent increase in interstitial reticulin observed by day 14 after administration. There is no description of observations made earlier than day 14 with this stain. At least one human fibrotic lung resulting from paraquat ingestion, evaluated by unspecified special stains,\(^2\) was reported to contain reticulin within the intra-alveolar spaces, presumably surrounding (and synthesized by) the invasive fibroblast-like cells within the alveolar space. Due to the extreme variability in treatment regimens and initial doses (as well as possible species specificity), it is difficult to

**FIGURE 3.** Representative sections prepared from lungs of rats administered 24 mg/kg of paraquat intraperitoneally and stained with hematoxylin/eosin. [a] Control rat, no damage evident (original magnification, \(\times 40\)). [b] Six days after paraquat administration, severe damage. Note obliteration of alveoli and dilatation of vessels and airways (original magnification, \(\times 40\)).

Six days after paraquat injection, marked widespread pathologic changes were seen (Fig 3). Much of the alveolar architecture was obliterated by cellular infiltration. Dilation of respiratory bronchioles and alveolar ducts was present, and the pleural mesothelium appeared hypertrophic and hyperplastic. Intense cellularity surrounding vessels and bronchioles was seen in contrast to the acellular edema observed at earlier times after paraquat administration. Despite the widespread damage, a few randomly distributed focal areas were seen in which alveolar architecture remained intact. Very intense intraalveolar, intraseptal, perivascular and peribronchial staining was noted with Gomori’s stain. An apparent excess of collagen fibers was visible in the subpleural region, as well as around large-sized vessels and airways. The results of these studies are summarized in Table 1.

**DISCUSSION**

At least two conclusions may be drawn from the data presented in this paper. First, in the animal model studied, injection of high doses of paraquat into rats caused acute pulmonary fibrosis. The
compare animal studies with human necropsies. In general, it seems that in both humans and rodents there is little interstitial collagen deposition despite severe pathologic changes elicited by paraquat.

Our histopathologic results are in broad agreement with the above-mentioned findings. In agreement with others (reviewed by Smith and Heath)\(^2\) we find early inflammatory pulmonary edema, especially peribronchial and perivascular edema,\(^18\) followed by later deposition of collagen fibers, in the presence of gross hypercellularity and obliteration of the alveolar architecture. We find that the collagen deposited (stained by van Gieson's or Masson's trichrome) tends to be predominantly peribronchial, perivascular, and subpleural, with less-dense staining of intraseptal and intra-alveolar collagen. An entirely unexpected finding was the continuously increasing staining with Comori's silver stain from day 1 after paraquat administration onwards. It is especially noteworthy that the material being visualized by Comori's stain has a different distribution within the lung than does that visualized by Masson's trichrome or van Gieson's stains, even though the argyrophilic material also first appears around vessels and airways. The intense argyrophilic staining at days 6 and 7 after paraquat, including strong intra-alveolar and intraseptal staining (which was first observable as early as day 2 after paraquat injection), was particularly striking. While the specificity of this particular stain is customarily assumed to be for reticulin (type III collagen) in normal tissue,\(^23,24\) further biochemical studies are necessary to document the nature of the material accumulating in the alveolar interstitium that is visualized by this particular stain in pathologic states (such as after paraquat administration). In particular, the possibility that newly synthesized fibers of type I collagen may be argyrophilic\(^24\) must be considered.

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