Concern has been expressed in regard to the possible toxic effects of hyperoxia on drug-sensitized lung tissue [1]. An increase in the toxicity of high concentrations of oxygen has been demonstrated in rodents after the administration of a variety of chemicals, including, for example, butylated hydroxytoluene [2]. For a number of compounds, such as paraquat and diquat, this has also been demonstrated in man [3]. In rodents, a similar degree of oxygen toxicity has been described in association with the combination of hyperoxia and the antineoplastic drug, bleomycin [4-8]. In man, treatment with bleomycin followed by high concentrations of oxygen is suspected of being a cause of pulmonary toxicity, as has been observed in bleomycin-treated patients after surgery [9]. This suspicion has led the anaesthetist to omit the use of hyperoxia during anaesthesia in bleomycin-treated patients, although doubt concerning the possible additive role of oxygen in bleomycin-induced pulmonary toxicity has been expressed [10, 11]. Since there is evidence that bleomycin exhibits its anti-tumour effect by generating oxygen radicals that disintegrate cellular DNA [12], the combination of bleomycin and high oxygen tensions may lead to superimposed toxicity.

In this respect, tissues with low bleomycin hydrolase activity, such as lung and skin, might be
most susceptible [13]. Agents which produce oxidative damage of the lung may stimulate lung defense mechanisms, which will limit injury as a result of a second insult by the same or a different agent. In this regard superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) may be important [14]. Apart from an augmentation of these enzymes after the first oxidative challenge, it is also possible that the activity decreases because of destruction of the enzymes and the enzyme synthetic machinery. If activated oxygen species are playing an important role in bleomycin-induced pulmonary toxicity with or without hyperoxia, alterations in the activities of SOD and GSH-Px may be expected.

The purpose of this study was to investigate pulmonary toxicity in rats after treatment with doses of bleomycin approaching those encountered clinically in the presence or absence of additional hyperoxia. Lung morphology was studied and the possible induction of protecting enzymes (SOD and GSH-Px) was assayed.

MATERIALS AND METHODS

Design of investigation

Female Wistar rats, bred under specific pathogen-free conditions (until weaning) were obtained from the Central Institute for the Breeding of Laboratory Animals (Zeist, The Netherlands). At the start of the investigation the animals were 11 weeks old and weighed 170 ± 10 g. They were housed in a laminar flow cabinet, at most three in a cage. They received sterilized rat chow (RMH-B Hope Farms, Woerden, The Netherlands) and acidified water ad libitum. After an adaptive phase of 5 weeks, i.p. injections of bleomycin sulphate 10 mg kg⁻¹ (Lundbeck, Copenhagen, Denmark) dissolved in 0.5 ml of sterile physiological saline, were performed in 11 rats three times weekly for a period of 6 weeks. Immediately before each injection the animals were weighed. Five control rats received physiological saline only. Before the first injection a chest radiograph of each rat was obtained under light ether anaesthesia. Nine weeks after the start of the injections a second chest radiograph was obtained. Four weeks after the termination of the bleomycin injections, seven bleomycin-treated rats were placed in an airtight Perspex cage and exposed for 4 h to 50% oxygen-enriched air. The flow was 4 litre min⁻¹, which was sufficient to prevent accumulation of carbon dioxide in the cage. The oxygen concentration was continuously measured with a Beckman oxygen analyser M 11. One week after the hyperoxia treatment all rats were anaesthetized (i.p. injection of 1.0 or 1.4 ml of a 6% solution of chloralhydrate for bleomycin-treated and controls, respectively) and a third chest radiograph obtained. Thereafter, the thorax was opened by a median incision and the trachea was cannulated. The left lung was ligated, excised and stored in ice cold physiological saline solution for the determination of SOD and GSH-Px activity. The right lung was perfused with pH 7.4 buffered 8% formalin solution for 20 min at 20 cm H₂O pressure and prepared for histology. The lungs of experimental and control rats were processed concomitantly.

Histology

After fixation, two parts of the right lung, each of approximately 0.5 cm³, were excised: a central part and a peripheral part of a “lower” lobe. Smaller pieces of each part were transferred into formalin for further fixation under vacuum and then embedded in glycolmethacrylate (Technovit 7100, Kultzer, Wehrheim, W. Germany). Two micrometre sections were cut and stained either with haematoxylin–eosin or with Bodia’s reticulin solution. Each section was presented “blind” to the examiner who screened microscopically 10 fields for oedema, fibrotic changes, accumulation of intra-alveolar macrophages and the presence of type II pneumocytes in the alveolar wall, using a 400x magnification. Extension of oedema, number of cells and the thickness of the alveolar wall all were compared for the controls, bleomycin-treated and bleomycin plus oxygen-treated groups. The rating was based on the subjective interpretation of one of us (R.V.), using a 0–3 grading system. A grade 1 lesion for fibrosis consisted of thickening of the interstitial septum by infiltration of fibroblasts, whereas a grade 3 lesion corresponded to a well established scar with prominent deposition of collagen. Regarding macrophages and type II pneumocytes, a grade 1 lesion represented one or more foci of increased numbers of cells, whereas a grade 3 lesion corresponded to numerous foci or pronounced proliferation of cells which obscured normal lung architecture. Oedema grade 1 consisted of a slight thickening of alveolar septa, whereas grade 3 corresponded to extra-alveolar transudate.
Biochemical determinations

A 20 % homogenate of excised pieces of the left lung (about 400 mg) was obtained with the aid of a glass homogenizer. Handling was performed at 4 °C. The homogenate was subjected to a two-step centrifugation procedure in an L3-40 Beckman centrifuge. The Ti50 rotor was used with Teflon adaptors for 2-ml cellulose nitrate tubes. The first step was a 30-min centrifugation at 13500 g directly followed in the same tube by 60 min at 131000 g. The supernatant was pipetted off, separated in three parts for the determination of SOD and GSH-Px activity and protein concentration.

The activity of SOD in the supernatant was assayed using a photochemical augmentation assay as described by Misra and Fridovich [15]. Briefly, the total reaction mixture was 3 ml containing riboflavin 13.3 μmol litre⁻¹ and di-anisidin 200 μmol litre⁻¹ as well as 0.1 ml of the supernatant in a quartz cuvette. The cuvette was placed in an illumination box midway between two parallel 20-W Sylvania Gro-Lux fluorescent tubes. The increase in absorbance at 460 nm was measured after 10 min of illumination. Enzyme activity was expressed as units per mg of protein.

As a standard a fresh preparation of bovine SOD (Sigma, 58254) was used containing 2700 units per mg of protein (E₂₅₅).

The activity of GSH-Px was assayed according to Lawrence and Burk [16]. Briefly, the total reaction mixture was 1 ml containing K₂HPO₄ 50 mmol litre⁻¹ (pH 7.0), EGTA 1 mmol litre⁻¹, NaN₃ 1 mmol litre⁻¹, NADPH 0.2 mmol litre⁻¹, GSH 1 mmol litre⁻¹, H₂O₂ 1 mmol litre⁻¹, GSSG reductase 1 E.U. and 0.1 ml of supernatant. The reaction was started by adding the hydrogen peroxide. Oxidation of NADPH was recorded as change in the absorbance at 340 nm. Enzyme activity was expressed as μmol of NADPH oxidized per mg of protein per min.

Statistics

For comparison of mean responses in the three groups the two-sided Student t test was used. The P values given for the histological and biochemical observations are those of a randomization version of the t test [17].

RESULTS

Regular microbiological control demonstrated the efficacy of the barrier created by the laminar flow cabinet. No microbiological contaminants were discovered in the lungs of the rats of any group during the experimental period. The average body weights of the rats during this period are shown in figure 1. The weight gradually increased before bleomycin treatment, and continued to increase in the control group. Rats receiving bleomycin showed a progressive decrease of their body weight, which started immediately after the first injection of bleomycin and continued even after the administration of bleomycin had been completed. The exposure to oxygen did not influence the slope of the curve.

Bleomycin-injected animals finally reached a body weight below their starting value.

The chest radiographs obtained at the three

![Fig. 1. Changes in mean body weight of 11 rats (●●●) after i.p. injection with bleomycin three times weekly for 6 weeks compared with those in five saline-injected controls (top: •••). Bleomycin injections started at the arrow. The second arrow points to the 4-h oxygen treatment.](image-url)
TABLE I. Data on lung histology of rats injected i.p. with bleomycin with or without oxygen treatment.
Scores (mean ± SD) as defined in methods. n = No. animals. *P < 0.05; **P < 0.01

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Macrophages</th>
<th>Pneumocytes II</th>
<th>Oedema</th>
<th>Fibrosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (n = 5)</td>
<td>0.3±0.2**</td>
<td>1.3±0.2</td>
<td>0.3±0.2</td>
<td>0.3±0.2</td>
</tr>
<tr>
<td>Bleomycin (n = 4)</td>
<td>0.8±0.2*</td>
<td>1.2±0.2</td>
<td>0.3±0.2</td>
<td>0.2±0.2</td>
</tr>
<tr>
<td>Bleomycin + oxygen (n = 7)</td>
<td>1.1±0.1**</td>
<td>1.1±0.1</td>
<td>0.6±0.2</td>
<td>0.5±0.2</td>
</tr>
</tbody>
</table>

*P < 0.05; **P < 0.01

Fig. 2. Photomicrograph of lung showing thickening of alveolar wall as a result of the replacement of type I pneumocytes by type II pneumocytes. The accumulation of macrophages (*) within an alveolus is shown. Horizontal bar represents 7 μm.

different time points revealed no pulmonary abnormalities.

Lung histology

Histological examination of the lungs demonstrated the presence of a mild reaction in all three groups of rats; that is, the mean scores were around one or less (table I). Only the number of intra-alveolar macrophages was significantly increased in both the bleomycin-treated and the bleomycin + oxygen-treated rats as compared with controls (fig. 2). The number of macrophages in the bleomycin + oxygen-treated rats was increased significantly when compared with bleomycin-treated animals. Foci of increased numbers of type II pneumocytes in the alveolar wall were observed incidentally, but these did not differ significantly in the three groups. Likewise, the other two variables did not differ significantly in the three groups of animals.
HYPEROXIA AFTER BLEOMYCIN TREATMENT

TABLE II. Effects of bleomycin injected i.p. with or without oxygen treatment on the activity of superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) in rat lung. Mean values ± SD (number of assays)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>SOD (u./mg protein)</th>
<th>GSH-Px (mol of NADPH/mg protein min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>6.5 ± 0.6 (4)</td>
<td>106 ± 15 (4)</td>
</tr>
<tr>
<td>Bleomycin</td>
<td>5.7 ± 0.8 (4)</td>
<td>89 ± 18 (4)</td>
</tr>
</tbody>
</table>
| Bleomycin plus 50 % oxygen     | 6.2 ± 0.9 (5)       | 103 ± 17 (5)                         | ns

Lung biochemistry

The activities of the enzymes SOD and GSH-Px determined for the three different groups of rats are presented in table II. These did not differ significantly between the three groups.

DISCUSSION

Rats injected with bleomycin 10 mg/kg body weight i.p. three times weekly for 6 weeks, demonstrated (apart from the decrease in body weight) one alteration in the lung consistent with bleomycin toxicity—intra-alveolar accumulation of macrophages. Exposure to oxygen significantly influenced only the bleomycin-induced macrophage response. The activities of the enzymes GSH-Px and SOD remained unaltered. Chest radiographs were normal. Aiolfi and colleagues [18] observed more serious symptoms in rats: hyperplasia of type II pneumocytes, thickening of alveolar septa, obliteration of alveolar spaces, desquamative alveolitis and beginning of fibrillogenesis after injection of bleomycin 10 mg kg⁻¹ i.p. every other day for 4 weeks. In addition, Tom and Montgomery [19] described more severe pulmonary changes in rats injected twice weekly with bleomycin 5 or 15 mg kg⁻¹ i.p. Schurig and co-workers [20], using bleomycin 24 mg kg⁻¹ s.c. three times weekly for 5 weeks, noted increases in alveolar macrophages and alveolitis, and interstitial pneumonitis and fibrosis in Sprague-Dawley rats. Similar effects were described for bleomycin in other animal species [21-23] after the parenteral administration of bleomycin.

Bleomycin-induced pulmonary damage was less marked in the present study. This, however, might create an adequate starting point for a superimposed oxygen response. Exposure to 50 % oxygen for 4 h, the approximate time span for anaesthesia during surgery, did not increase lung toxicity in our rats. Thus 50 % oxygen delivered for 4 h was not a complicating factor for bleomycin as regards the production of pulmonary toxicity in our rats. This finding is contrary to the results of a number of investigators who exposed animals to oxygen during, or subsequent to, treatment with bleomycin and obtained additive or potentiated toxic activity. Thus, Toledo and co-workers [4] found potentiation of bleomycin toxicity in mice that received 40 mg kg⁻¹ s.c. twice weekly during 10 weeks of continuous exposure to 40 % oxygen. Exposure of hamsters to 70 % oxygen for 3 days subsequent to intratracheal administration of bleomycin decreased its LD₅₀ from 7.3 to 2.3 mg kg⁻¹ [6]. In all animals interstitial pneumonitis and fibrosis was present, and these were most severe in the oxygen-treated groups. Rinaldo, Goldstein and Snider [8] established increased interstitial injury in hamsters intratracheally instilled with bleomycin before exposure to either 60 or 100 % oxygen for 21 days. The degree of lung injury turned out to be related to the oxygen concentration and to be inversely related to the time elapsing between the instillation of the bleomycin and the start of the exposure to oxygen. Potentiation of bleomycin 50–100 mg kg⁻¹-induced lung damage by exposure to 70 % oxygen for 8 days was observed in mice if this exposure started immediately after the i.p. injection of bleomycin [5]. Finally, the combination of intratracheal bleomycin 6.0 mg kg⁻¹ and 100 % oxygen for 48 h after instillation of the drugs resulted in synergistic alveolar toxicity in rats [24].

In all these studies the dose of oxygen delivered to the animals was greater than we used and exceeded the amount administered under clinical conditions. In most instances the dose of bleomycin was also greater; certainly, the dose reaching the lungs after intratracheal instillation of bleomycin was higher. It was determined that,
after injection of bleomycin 10 mg kg\(^{-1}\) s.c. to mice, the maximal pulmonary concentration was less than 1% of the injected dose [25]. If a comparison is allowed with our rats injected with bleomycin i.p., approximately 0.02 mg reached the lungs after each injection. This dose was described to be effective in rats in causing several symptoms of lung toxicity [18] if injected every other day for 4 weeks. The total dose in this way exceeded that usually administered clinically. However, patients receive bleomycin by i.v. infusion, which is certainly more effective.

A possible explanation for the minor histological deviation from the control state observed in our study might be the induction of defense mechanisms during exposure to bleomycin. This, however, was not reflected in any increase in the activities of the antioxidant enzymes SOD and GSH-Px. This result questions the role of activated oxygen species in bleomycin toxicity. Phan and Fantone [26] presented evidence that the inhibition of bleomycin-induced pulmonary toxicity by lipopolysaccharide did not correlate with effects on antioxidant enzymes.

Our results with the dose of bleomycin used, in combination with the realistic oxygen exposure, are not totally unexpected in the light of the results of LaMantia, Glick and Marshall [11] obtained in man. These authors demonstrated that oxygen was not an adjuvant factor to bleomycin-induced toxicity. In fact, they confirmed the retrospective study of Douglas and Coppin [10] who found but one non-fatal case of pulmonary complications in 20 surgical patients previously treated with bleomycin in doses ranging from 150 to 570 mg and receiving, during surgery, oxygen concentrations up to 100%.

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