

Effect of oxygen on bleomycin-induced lung damage

Ramesh N. Sogal, MD
Alexandru A. Gottlieb, MD
Azmy R. Boutros, MD
Ram R. Ganapathi, PhD
Raymond R. Tubbs, MD
Patricia Satariano, CRNA
Paul R. Blakely, CRNA
Gerald J. Beck, PhD

Mortality and lung damage resulting from bleomycin-oxygen interaction were studied in mice. No animals died in the control group given saline and breathing 21% to 50% oxygen. Mortality following injection of 10 mg/kg and 20 mg/kg bleomycin, respectively, increased from 0% and 6% following exposure to 21% oxygen, to 20% and 33% after 30% oxygen, and to 47% and 66% after 50% oxygen. Pulmonary pathology also became progressively more severe at the higher oxygen concentrations as measured by pulmonary hydroxyproline content and a visually scored index of cellular pathology.

Index terms: Bleomycin • Lung, injury • Oxygen

Cleve Clin J Med 1987; 54:503-509

Bleomycin-induced pulmonary damage may be aggravated by hyperoxia; this interaction has been reported in patients¹ and confirmed in laboratory animals.²⁻⁶ However, recommendations to restrict inspired oxygen to near-normal concentrations perioperatively⁷⁻¹² have been contested by retrospective clinical analyses.^{13,14} We therefore carried out further laboratory studies, which show a graded increase in mortality and associated pulmonary histopathology following exposure of bleomycin-treated mice to progressively higher oxygen concentrations.

Methods

One hundred eighty female, white, Swiss Webster mice, weighing 20 to 25 g each, were divided into three groups and were injected subcutaneously twice weekly for five weeks as follows: group I with saline; groups II and III

Division of Anesthesiology, The Cleveland Clinic Foundation. Submitted for publication October 1986; accepted Jan 1987; revision received June 1987.

0891-1150/87/06/0503/07/\$2.75/0

Copyright © 1987, The Cleveland Clinic Foundation

Table. Oxygen exposure and mortality

Treatment	Oxygen exposure	Mortality*
Saline	all groups	0
Bleomycin 10 mg/kg	21%	0
	30%	3
	40%	3
	50%	7
20 mg/kg	21%	1
	30%	5
	40%	10
	50%	10

* Mortality (numbers) following exposure to various inspired oxygen concentrations in groups of 15 mice injected with saline (control) or bleomycin 10 mg/kg or 20 mg/kg. (Mortality trends by generalized Wilcoxon rank sum test: $P < .01$ for both doses.)

with bleomycin, 10 and 20 mg/kg respectively. The three groups were each subdivided into four subgroups of 15 mice each. Following subcutaneous drug injection (twice weekly), the subgroups breathed air (21% oxygen) or 30%, 40%, or 50% oxygen for 48 hours in a specially designed oxygen chamber. The study was extended for two weeks after the final injection; thus, for each animal there were 10 injections in all and 14 exposures to air or high oxygen. Surviving mice were killed by cervical fracture. The anterior thorax was opened and the right mainstem bronchus exposed and clamped with a

hemostat. The right lung was then excised at the hilus and frozen immediately for hydroxyproline assays.^{15,16} The left lung was insufflated with 10% neutral buffered formalin by intratracheal injection. The expanded lung was excised, immersed in neutral buffered formalin for 24 hours, and embedded in paraffin following dehydration in graded alcohols. The 6- μ m rehydrated sections were stained with hematoxylin and eosin.

Analysis of hydroxyproline

The hydroxyproline content of the lung was determined as described previously.^{15,16} The wet lung weights were determined and the minced samples hydrolyzed in 6N HCl (1.0 mL/0.1 g of lung) at 130°C for three hours. After the hydrolysates were cooled, the theoretical amount of 2.5N NaOH to neutralize the 6N HCl was added to them. Samples were then neutralized to pH 6.8–7.0 using dilute solutions of 0.1N NaOH. Neutralized hydrolysates were filtered using Whatman #1 paper and diluted to 25 mL using distilled water.

Hydroxyproline standards were prepared fresh daily in 2.0-mL volumes containing 1 μ g to 5 μ g of hydroxyproline. Each standard was prepared in duplicate, the second serving as a blank substrate. Duplicate aliquots of samples (2.0 mL or a dilution made up in 2.0 mL) were set up in 16, 100-mm Pyrex tubes. Dilution of samples was necessary to maintain hydroxyproline values in a 1–2 μ g range. Reagents in 1-mL volumes were

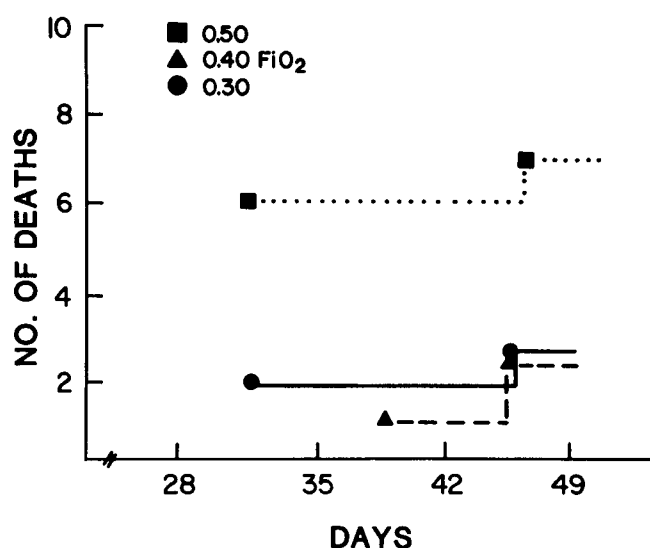


Fig. 1. Cumulative deaths in subgroups of mice ($n = 15$ for each subgroup) given bleomycin 10 mg/kg and exposed to 30%, 40%, or 50% oxygen (FiO_2 0.30, 0.40, or 0.50).

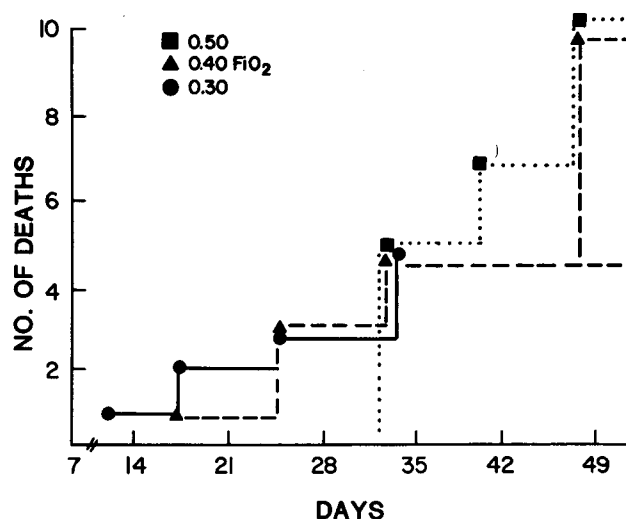


Fig. 2. Cumulative deaths in subgroups of mice ($n = 15$ for each subgroup) given bleomycin 20 mg/kg and exposed to 30%, 40%, or 50% oxygen (FiO_2 0.30, 0.40, or 0.50).

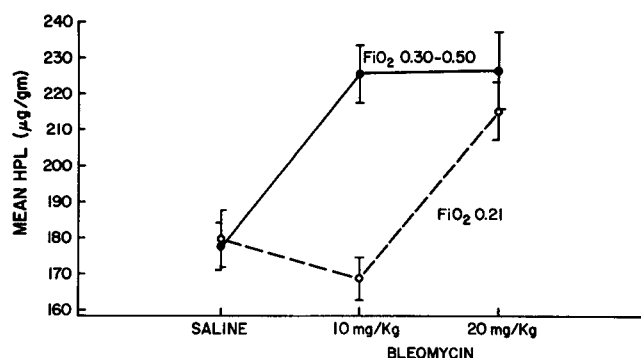


Fig. 3. Mean hydroxyproline (HPL) concentrations ($\mu\text{g/g}$) in lungs of mice treated with 10 mg/kg or 20 mg/kg bleomycin and exposed to atmospheric air (21% oxygen, FiO_2 0.21) (---) or oxygen in the range 30%–50% (FiO_2 0.30–0.50) (—).

added to samples and standards in the following order: chloramine T, 0.05M; perchloric acid, 3.15M (except for blanks, perchloric acid was added before chloramine T); and p-dimethylaminobenzaldehyde 20% solution, followed by a 20-minute incubation in a 60°C water bath. After a five-minute cooling period, absorbance at 557 nm of standards and samples followed by their respective blanks was determined in the percent transmittance mode using an Aminco-Bowman Ratio II spectrofluorometer. Hydroxyproline values were expressed as micrograms hydroxyproline per gram lung.

Histopathology

Histopathologic assessment was done without knowledge of drug or oxygen conditions for each specimen. A semiquantitative grade (0, 1, 2, 3, 4) was assigned for several variables on each tissue sample. The histologic evaluations included cuboidal alveolar metaplasia, interstitial edema, interstitial lymphocytic and histiocytic infiltration, free alveolar macrophage accumulation (FAM), fibrin hyaline membrane formation, endothelial proliferation, necrosis, interstitial fibrosis, and pleural inflammation and fibrosis. Of these, the most easily and reproducibly identified were the accumulation of free alveolar macrophages and interstitial lymphocytes and histiocytes, collectively referred to as the cumulative cellularity index (CCI). The other factors varied widely, were often focally distributed throughout the lung parenchyma, and were more difficult to recognize and quantify. For this reason, FAM and CCI scores were used to compare pathologic findings between groups of animals and to derive

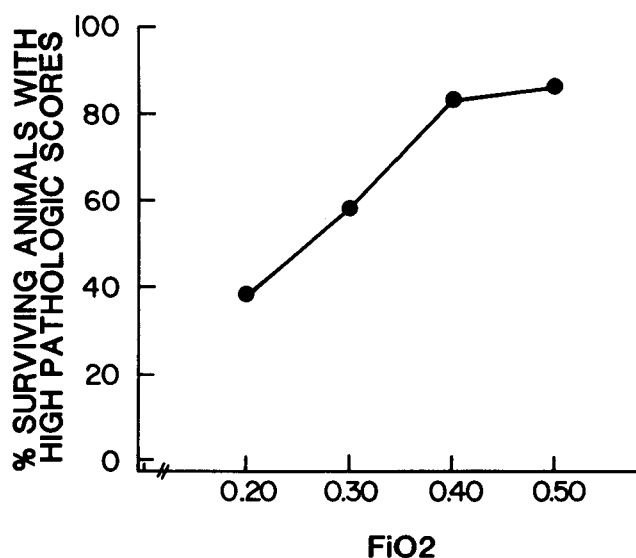


Fig. 4. Percentage of surviving mice exposed to 10 mg/kg bleomycin and having high pathologic scores, against oxygen exposure, 20%–50% (FiO_2 0.21–0.50).

a visual assessment of cellular pathology (FAM/CCI). Also, to allow for the uneven attrition by the end of the study, a “severity index” (see Discussion) was derived for each experimental subgroup and defined as proportion of tissue specimens with high scores (2, 3, or 4) times the percentage mortality.

Mortality curves for all groups were compared using the generalized Wilcoxon rank sum test.¹⁷ A two-sample t-test was also used for the air versus oxygen groups and to compare mean hydroxyproline concentrations.

Results

No animals in the control group died after saline injection and subsequent exposure to increasing concentrations of oxygen. The overall mortality rates (attributable to drug plus oxygenation) for bleomycin 10 and 20 mg/kg were 22% and 43%, respectively. The mortality curves over the follow-up period were significantly different (generalized Wilcoxon test) for control versus bleomycin ($P < .001$), and between drug doses ($P < .01$).

The influence of oxygen exposure (FiO_2 0.30, 0.40, 0.50) on mortality after bleomycin administration is enumerated in the Table. At both drug doses, the adverse effect of oxygen concentrations was highly significant ($P < .01$). After bleomycin 10 mg/kg was administered, mortality was

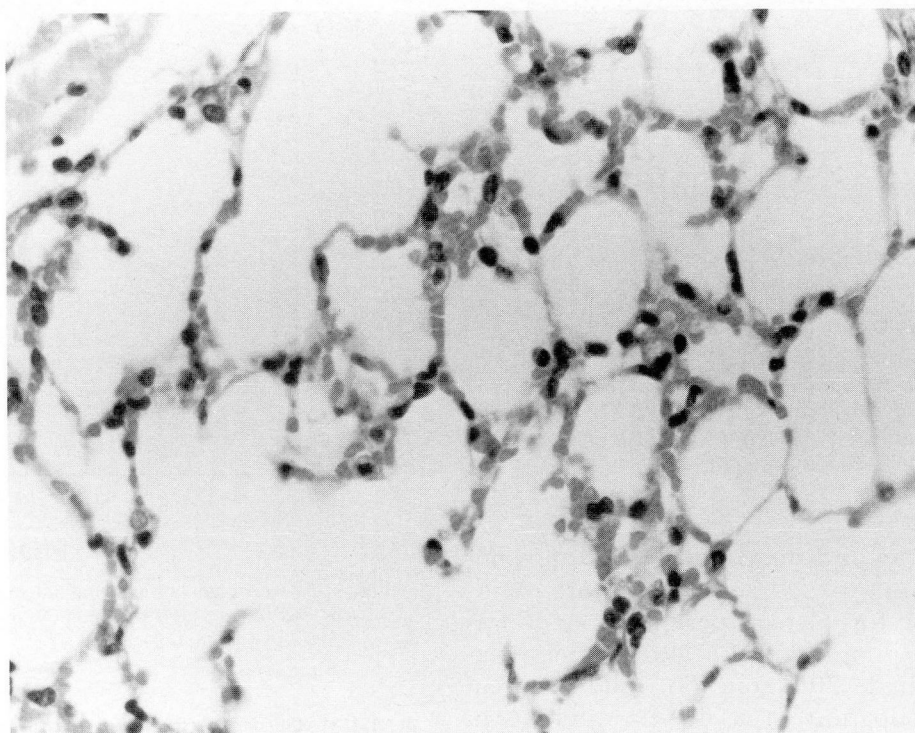


Fig. 5. Insuflated mouse lung (control group). No significant histopathologic changes were identified. (Hematoxylin and eosin $\times 400$)

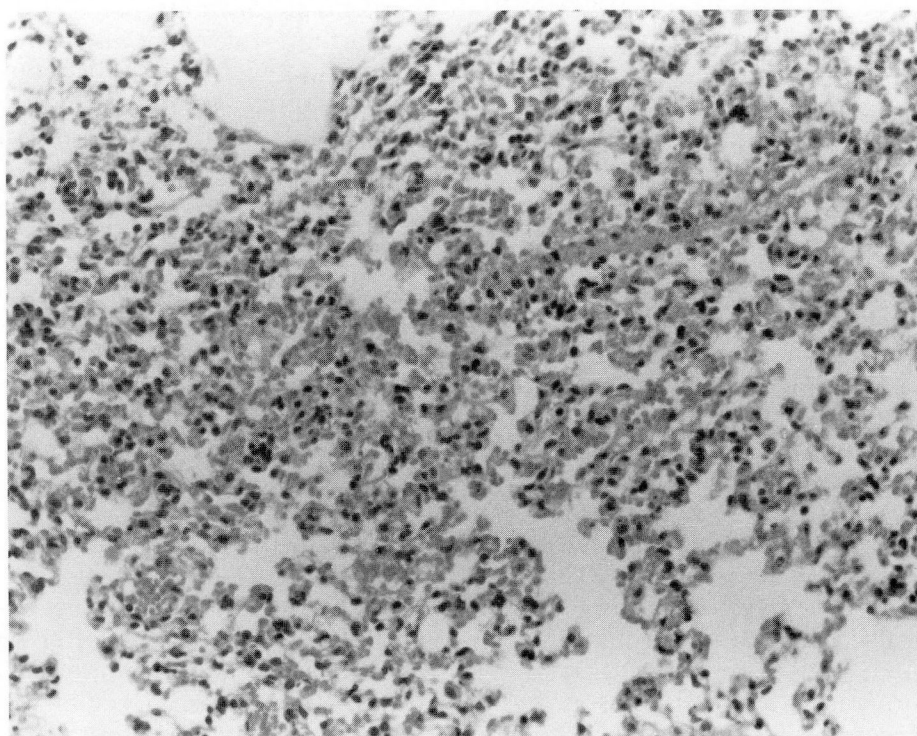


Fig. 6. Insuflated mouse lung after bleomycin and 50% oxygen. The alveolar septae are expanded by mononuclear cells and free alveolar macrophages have accumulated in the alveolar space. (Hematoxylin and eosin $\times 400$)

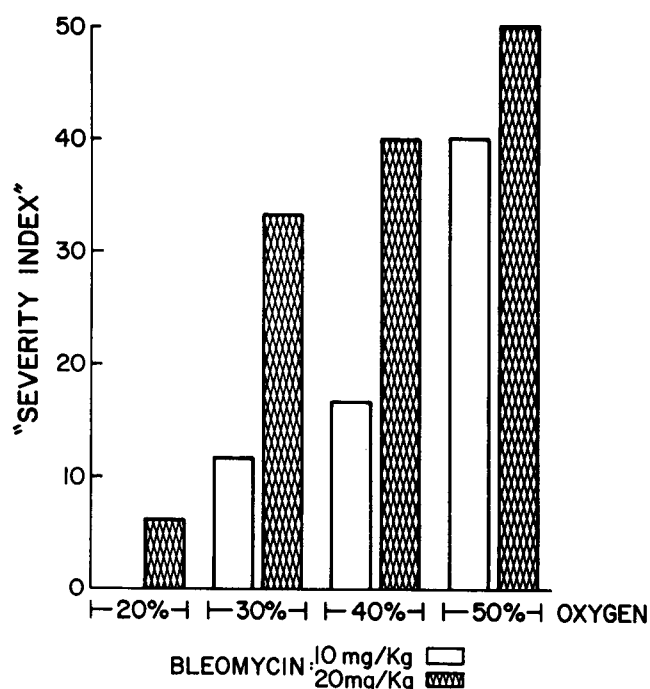


Fig. 7. "Severity Index" (see text) of lung pathology related to oxygen exposure and bleomycin dose.

20% for both FiO_2 0.30 and 0.40, increasing to 47% at FiO_2 0.50. For 20 mg/kg bleomycin, the death rate reached 66% after exposure to FiO_2 0.40 or 0.50. Mortality differences within the high-oxygen groups (FiO_2 0.30, 0.40, or 0.50) just missed statistical significance ($P > .05$) following the higher dose. The incremental death rates at weekly intervals for the two bleomycin doses and the three high-oxygen exposures are plotted in Figures 1 and 2.

The bleomycin-treated animals were lethargic, lost weight, and showed hair loss, circumoral ulcers, and increased salivation.

Mean levels of hydroxyproline ($\mu\text{g/g}$ lung \pm S.E.) are plotted in Figure 3 in relation to the two drug doses. Bleomycin 10 mg/kg, associated with air breathing, did not change the hydroxyproline level. The combined effect of this dose and oxygenation, however, was to increase the mean hydroxyproline level to about the same degree as did bleomycin 20 mg/kg, with or without oxygen enrichment. The increases were highly significant ($P < .01$), as were the differences between the levels associated with air versus oxygen breathing at the low dose of drug.

For the lower bleomycin dose, there was a

progressive relation between exposure to increasing FiO_2 and the proportion of surviving animals with (unadjusted) high pathologic scores (Fig. 4). For example, 86% of mice showed high scores after breathing 50% oxygen compared with 38% for those exposed to atmospheric air. The other histopathologic studies were impeded by the loss of specimens due to mortality, which was especially severe following bleomycin 20 mg/kg. The "severity index," which was derived in order to take this into account, is referred to in the Discussion section.

Figures 5 and 6 illustrate, respectively, the normal pulmonary histology in the control group of mice, and the pathological changes associated with bleomycin and FiO_2 0.50.

Discussion

Our results confirm that increased mortality is associated with hyperoxia in bleomycin-treated laboratory animals, that pulmonary damage is similarly worsened, and that these effects can be correlated with the inspired oxygen concentration.

For technical reasons (see Methods section), studies of lung pathology were not possible when animals died before the investigation was completed. Since the resulting loss of information hampered statistical analysis of the histopathological data, especially following the 20 mg/kg dose, we devised a "severity index" into which mortality was incorporated. The validity of this index, which is based on a visually scored assessment of cellular pathology (FAM/CCI) and the proportion of deaths in each subgroup, rests on the assumption that pulmonary toxicity is responsible for mortality. The evidence for this (in the absence of comprehensive testing of pulmonary function in larger laboratory animals) comes from studies similar to our own; however, it has also been affirmed that ventilatory failure is the immediate cause of death in mice exposed to one or more atmospheres of oxygen¹⁸ (without bleomycin).

In Figure 5 we have plotted "severity index" against FiO_2 for bleomycin 10 mg/kg and 20 mg/kg to confirm the graded relationship shown for the unadjusted pathologic scores in respect to the lower dose (Results, Figure 4). The index showed, as did the hydroxyproline levels, that bleomycin 10 mg/kg combined with hyperoxygenation caused at least as much lung damage as the higher dose of the drug alone. That this conclusion is

conservative is suggested by comparing FiO_2 of 0.40 and 0.50 following bleomycin 10 mg/kg with FiO_2 of 0.21 and 0.30 after the 20 mg/kg dose, the respective mortality rates being 33% and 20%.

Although our findings point to a progressive worsening of pulmonary pathology proportionate to FiO_2 , differences in mortality between the oxygen groups did not reach statistical significance, which may suggest that an adverse effect is possible from any degree of overoxygenation.

Reference must be made to the conflict between studies such as ours and those, mainly clinical, that do not show a pathological interaction between bleomycin and oxygen administration.¹³ Certain authors, while apparently accepting the adverse effect of added oxygen during anesthesia, have not found pulmonary morbidity either when inspired oxygen was kept below 25%¹⁰ or in a series of 14 patients when it was in the range 30–100%.⁹ Nygaard et al,¹⁹ publishing almost coincidentally with and apparently confirming the findings of Goldiner et al,¹ made no mention of hyperoxia, referring to “surgical trauma” as the cause of respiratory failure in their series. Also Goldiner and Rooney,¹¹ in an editorial comment, have referred to “more than 700 bleomycin-treated patients” who underwent surgery and had inspired oxygen concentrations limited to less than 30% and with closely monitored fluid replacement; in their words: “We have seen no postoperative pulmonary failure in this group of patients.”

Factors explaining such apparently contradictory findings probably include the time and duration of the bleomycin-oxygen interaction, the respective doses and concentrations, and the possibility that lesser degrees of pulmonary damage may escape clinical notice or be attributed to other causes.

Meanwhile, it seems only prudent not to expose bleomycin-treated patients to oxygen concentrations above about 25% unless these are essential to support life.

Acknowledgments

We thank Dr. Ronald A. Millar for his assistance with the manuscript, and Bristol Laboratories for supplying the bleomycin for the study.

References

- Goldiner PL, Carlon GC, Cvitkovic E, Schweizer O, Howland WS. Factors influencing postoperative morbidity and mortality in patients treated with bleomycin. *Brit Med J* 1978; **1**:1664–1667.
- Toledo CH, Ross WE, Hood CI, Block ER. Potentiation of bleomycin toxicity by oxygen. *Cancer Treat Rep* 1982; **66**:359–362.
- Hakkinen PJ, Whiteley JW, Witschi HR. Hyperoxia, but not thoracic x-irradiation, potentiates bleomycin- and cyclophosphamide-induced lung damage. *Am Rev Respir Dis* 1982; **126**:281–285.
- Tryka AF, Skornik WA, Godleski JJ, Brain JD. Potentiation of bleomycin-induced lung injury by exposure to 70% oxygen. Morphologic assessment. *Am Rev Respir Dis* 1982; **126**:1074–1079.
- Rinaldo J, Goldstein RH, Snider GL. Modification of oxygen toxicity after lung injury by bleomycin in hamsters. *Am Rev Respir Dis* 1982; **126**:1030–1033.
- Berend N. The effect of bleomycin and oxygen on rat lung. *Pathology* 1984; **16**:136–139.
- Goldiner PL, Schweizer O. The hazards of anesthesia and surgery in bleomycin-treated patients. *Semin Oncol* 1979; **6**:121–124.
- Hulbert JC, Grossman JE, Cummings KB. Risk factors of anesthesia and surgery in bleomycin-treated patient. *J Urol* 1983; **130**:163–164.
- Douglas MJ, Coppin CML. Bleomycin and subsequent anaesthesia: A retrospective study at Vancouver General Hospital. *Can Anaesth Soc J* 1980; **27**:449–452.
- Allen SC, Riddell GS, Butchart EG. Bleomycin therapy and anaesthesia. *Anaesthesia* 1981; **36**:60–63.
- Goldiner PL, Rooney SM. In defense of restricting oxygen in bleomycin-treated surgical patients. *Anesthesiology* 1984; **61**:225–226.
- Gilson AJ, Sahn SA. Reactivation of bleomycin lung toxicity following oxygen administration: A second response to corticosteroids. *Chest* 1985; **88**:304–306.
- LaMantia KR, Glick JH, Marshall BE. Supplemental oxygen does not cause respiratory failure in bleomycin-treated surgical patients. *Anesthesiology* 1984; **60**:65–67.
- LaMantia KR, Glick JH, Marshall BE. Correspondence. *Anesthesiology* 1984; **61**:226–227.
- Sikic BI, Young DM, Mimnaugh EG, Gram TE. Quantification of bleomycin pulmonary toxicity in mice by changes in lung hydroxyproline content and morphometric histopathology. *Cancer Res* 1978; **38**:787–792.
- Woessner JF. The determination of hydroxyproline in tissue and protein samples containing small proportions of this amino acid. *Arch Biochem Biophys* 1961; **93**:440–447.
- Breslow N. A generalized Kruskal-Wallis test for comparing K samples subject to unequal patterns of censorship. *Biometrika* 1970; **57**:579–594.
- Winter PM, Smith G. The toxicity of oxygen. *Anesthesiology* 1972; **37**:210–241.
- Nygaard K, Smith-Erichsen N, Hatlevoll R, Refsum SB. Pulmonary complications after bleomycin, irradiation and surgery for esophageal cancer. *Cancer* 1978; **41**:17–22.
- Ramesh N. Sogal, MD
Division of Anesthesiology
The Cleveland Clinic Foundation
9500 Euclid Avenue
Cleveland, Ohio 44106