PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE **186**, 327–332 (1987)

# Effects of Intratracheal Instillation of Bleomycin on Phospholipid Synthesis in Hamster Lung Tissue Slices[1] (42621)

SHRI N. GIRI

*Department of Veterinary Pharmacology and Toxicology, School of Veterinary Medicine, University of California, Davis, California 95616*

*Abstract.* Bleomycin, an antineoplastic drug, is known to produce interstitial pulmonary fibrosis (IPF). As a result, it is commonly employed in various species to produce animal models of fibrosis. We have examined the uptake of [$^{14}$C]acetate by lung slices and its incorporation into lipids in the slices at various times following intratracheal administration of a fibrogenic dose (10 units/kg) of bleomycin in hamsters. As compared to saline controls, bleomycin had no effect on [$^{14}$C]acetate uptake at 4 and 7 days but it increased the uptake at 2 and 14 days after treatment. The incorporation of [$^{14}$C]acetate into total lipid was significantly reduced to 44, 62, 62, and 75% of the control at 2, 4, 7, and 14 days after bleomycin treatment, respectively. The incorporation into lipid as a percentage of the uptake was 12.2 in control animals whereas in bleomycin-treated animals, it was 4.7, 8.0, 7.3, and 6.9 at the corresponding times. Separation of lipids into various fractions revealed that bleomycin treatment specifically inhibited the synthesis of phosphatidylcholine and neutral lipid at all times of the study. The synthesis of all other phospholipids except phosphatidylethanolamine was depressed at 2 days. The latter was, however, depressed at 7 and 14 days after bleomycin treatment. It was concluded from the present study that bleomycin treatment inhibits the synthesis of phospholipid and neutral lipid and this may eventually lead to decreased surfactant production. 

Bleomycin, a mixture of glycopeptides, is therapeutically used for treatment of selected human cancers in combination with other antineoplastic agents and radiotherapy (1–3). One of the serious adverse side effects of this anticancer drug is that it produces interstitial pulmonary fibrosis (IPF) in a dose-related fashion (4, 5). As a result intratracheal administration of bleomycin is commonly employed to produce an animal model of IPF (6–9) since the histopathologic and biochemical features of the model are similar to IPF seen in humans (7, 9, 10). The biochemical mechanisms responsible for bleomycin-induced lung fibrosis are not clearly understood. It is known, however, that bleomycin is capable of generating reactive oxygen species (ROS) such as superoxide and hydroxyl radicals in the presence of molecular oxygen (11–13). The availability of excess ROS is shown to cause DNA strand scission (14, 15) and lipid peroxidation of the cell membrane (16, 17).

Microscopic studies of the lung from mice treated with bleomycin revealed that the primary lesions were endothelial swelling, interstitial edema, thickening of the alveolar wall, and proliferation of type II pneumocytes containing giant lamellar bodies (18). The lamellar bodies are considered to be the main storage and secretory granules of surfactant in type II cells of the lung (19).

There are conflicting reports in the literature with respect to changes in the level of surfactant in the lung of bleomycin treated animals. According to Burkhardt (20) and Gebbers (21) the pathogenic basis for bleomycin-induced lung damage is a reduction in lamellar bodies and consequent surfactant deficiency. This is diametrically opposed to the findings of Fasske and Morgenroth (18) who reported proliferation of type II pneumocytes with overproduction of surfactant. However, in none of the above studies was the synthesis of phospholipids, a major component of the lung surfactant, biochemically assessed.

[1] This work was supported by Grant 5RO1 HL2735-06 from the National Heart, Lung and Blood Institute of the National Institute of Health, Bethesda, Maryland.



0037-9727/87 $1.50

In view of this uncertainty, the objective of the present study was to evaluate the various stages involved in the *de novo* synthesis of phospholipids and neutral lipids from [$^{14}$C]-acetate in lung slices of hamsters treated with a fibrogenic dose of bleomycin. The evaluation of various stages included (i) the uptake of [$^{14}$C]acetate into lung slices and (ii) the incorporation of [$^{14}$C]acetate into total lipids as well as into various lipid fractions including phosphatidylcholine, a major constituent of surfactant and an important structural component of the lung membrane.

**Materials and Methods.** Male Golden Syrian hamsters weighing 90 to 120 g were purchased from Simonsen, Inc. (Gilroy, CA). They were housed in groups of four in plastic cages and had access to tap water and laboratory chow *ad libitum*. Bleomycin sulfate (Blenoxane) was a gift from Bristol Laboratories (Division of Bristol Myers Co., Syracuse, NY). Sodium acetate (1-$^{14}$C, sp act 54 mCi/mmol) was purchased from Amersham Corporation (Arlington Heights, IL). Precoated $K_6$ silica gel plates 40 Å (250-$\mu$ thick) were obtained from Whatman, Inc. (Hillsboro, OR). Standards for neutral lipid and different phospholipids were from Sigma Chemical Co. (St. Louis, MO). Dulbecco's modified Eagle's medium (DMEM) with Hepes buffer was purchased from GIBCO Laboratories (Chagrin Falls, OH). All other chemicals and solvents were from standard commercial sources.

*Treatment of animals.* Hamsters were acclimated for at least 1 week in the laboratory housing facilities prior to use in the experiment. They were anesthetized with sodium pentobarbital (80–90 mg/kg ip). Bleomycin sulfate freshly dissolved in sterile isotonic saline was instilled into the lung by transoral route under anesthesia. The dose of bleomycin was 1 unit in 0.5 ml saline per 100 g of body weight or an equivalent volume of saline in control hamsters. The hamsters were killed by exsanguination under anesthesia at 2, 4, 7, and 14 days after treatment. The lungs were perfused with oxygenated ice-cold isotonic saline through the right side of the heart *in situ*. All lung lobes from each animals were excised, cleaned of the extraneous tissues, rinsed in oxygenated ice-cold saline three times, and then transferred into a beaker containing ice-cold oxygenated DMEM. Using a Stadie-Riggs Microtome, slices of uniform thickness (0.5 mm) were cut from all lung lobes from each animal in a cold room at 4–6°C. The slices of each animal were pooled into a beaker containing DMEM. After gentle rinsing, the slices from each hamster's lung were transferred into a 25-ml Erlenmeyer flask containing 9 ml of DMEM pregassed with 5% $CO_2$ + 95% $O_2$ (v/v). One milliliter of the same medium containing 5 $\mu$Ci [1-$^{14}$C]acetate was added to each flask sitting on ice. After mixing, 25 $\mu$l of the medium was removed for determination of radioactivity. The flasks were incubated at 37°C in a Dubnoff metabolic shaker for 3 hr under an atmosphere of 5% $CO_2$ + 95% $O_2$. At the end of the incubation period an aliquot of 25 $\mu$l of the medium was removed for determination of radioactivity. The difference in radioactivity of the medium between pre- and postincubation was a measure of the [$^{14}$C]acetate uptake by the slices (22). The slices, after removal from the incubation flask, were washed three times with saline and then stored at −20°C.

The thawed lung slices from each animal were homogenized in 10 ml of chloroform:methanol (2:1, v/v) using a Polytron (Brinkmann Instruments, NY). $H_2O$ (2 ml) was added to the homogenate to create a two-phase system. After mixing and centrifugation, the chloroform layer, which contained the lipid material, was aspirated and transferred into a tube. Chloroform was evaporated to dryness under a stream of nitrogen. The residual lipid layer was redissolved in 250 $\mu$l of chloroform:methanol (2:1, v/v) and then the total volume was recorded. A 100-$\mu$l aliquot was used to measure the radioactivity and 10 $\mu$l was spotted onto precoated $K_6$ silica gel plates (250 $\mu$). A mixture of sphingomyelin (SM), phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylethanolamine (PE), and tripalmitin (neutral lipid, NL) was also spotted onto the plates. The plates were developed to a height of 15 cm in a solvent system consisting of chloroform:methanol:acetic acid:water (50:37.5:3.5:2), air dried, and sprayed with a saturated solution of fluorescein in methanol:water (1:1, v/v). This is a modification of

the method of Skipski *et al.* (23). Once the plates were air dried, the spots were visualized under uv light after exposure to ammonium hydroxide vapor. The bands containing the various lipid fractions corresponding to authentic lipid standards were scraped into vials and used for determination of radioactivity.

*Determination of radioactivity.* In order to determine radioactivity, 15 ml Tritosol scintillation cocktail (24) was added to each vial. After mixing, the vials were left at room temperature overnight to get rid of any chemiluminescence and then counted in a Model LS5801 Beckman scintillation spectrometer at a setting of 2% 2-$\sigma$. The cpm of each sample was automatically converted to dpm by a quench curve programmed in the counter. The quench curve was prepared with [$^{14}$C]-toluene and varying amounts of chloroform. The counting efficiency of the samples ranged from 85 to 90%.

*Presentation and statistical analysis of the data.* The data are expressed per total lung per 100 g body weight. Expression of the data on a per lung basis rather than per milligram wet weight, per milligram protein, or DNA avoids the artifactual lowering of the values in bleomycin-treated animals (25, 26) since this treatment causes the leakage of plasma proteins and infiltration of leukocytes into the lung and they cannot be completely washed out by *in situ* perfusion of the lung. The values are reported as the mean ± standard error (SE). Statistical significance was determined by using unpaired *t* test. *P* values $\leq 0.05$ were considered significant.

**Results.** The effect of intratracheal administration of bleomycin on [$^{14}$C]acetate uptake by lung slices is shown in Table I. The uptake of [$^{14}$C]acetate by lung slices from bleomycin-treated animals remained unaltered as compared with the control except that the uptake was increased to 114 and 132% of control at 2 and 14 days after treatment. As shown in Table II, the incorporation of [$^{14}$C]acetate into total lipid of lung slices was significantly reduced to 44, 62, 62, and 75% of control at 2, 4, 7, and 14 days after intratracheal instillation of bleomycin. The incorporation into lipid expressed as percentage of uptake was 12.2% in lung slices from control hamster whereas in bleomycin-treated animals, the incorporation was 4.7, 8, 7.3, and 6.9% of the uptake at 2, 4, 7, and 14 days after treatment, respectively (Table II).

TABLE I. EFFECT OF INTRATRACHEAL INSTILLATION OF BLEOMYCIN (Bleo) (10 units/kg) ON THE UPTAKE OF [$^{14}$C]ACETATE BY LUNG SLICES

| Time after treatment (day) | Uptake of [$^{14}$C]acetate (dpm/lung/100 g body wt) × $10^5$ | *P* value between control and Bleo |
|---|---|---|
| Control[a] | 43.7 ± 1.7[b] (10)[c] | — |
| Bleomycin | | |
| 2 | 50.0 ± 2.1 (6) | <0.05 |
| 4 | 40.3 ± 1.9 (6) | NS[d] |
| 7 | 52.5 ± 5.7 (6) | NS |
| 14 | 57.8 ± 21.9 (5) | <0.001 |

[a] Control values at 2, 4, 7, and 14 days after intratracheal injection of isotonic saline have been pooled since the values were not different.
[b] Each value represents the mean ± standard error.
[c] Number in parentheses is the number of animals.
[d] NS, not significant.

The second phase of the study was designed to show the effect of bleomycin treatment on the synthesis of specific lipids. This objective was accomplished by fractionating total lipid into various fractions by thin layer chromatography. As shown in Table III, [$^{14}$C]acetate incorporation into phosphatidylcholine of lung slices from bleomycin-treated animals was significantly depressed at all times as compared with the control. The inhibitory effect of bleomycin treatment on incorporation of [$^{14}$C]acetate into sphingomyelin, phosphatidylserine, and phosphatidylinositol was seen only at 2 days after treatment. The incorporation into phosphatidylethanolamine was depressed at 4 and 7 days, whereas into neutral lipid it was depressed at all times except 7 days after bleomycin treatment.

**Discussion.** The data collected in this study indicate that intratracheal administration of bleomycin decreased the incorporation of [$^{14}$C]acetate into some phospholipids, specifically phosphatidylcholine and neutral lipid. One of the mechanisms which may account for decreased synthesis of phosphatidylcholine and neutral lipids may be a decreased uptake of [$^{14}$C]acetate by the lung slices of bleomycin-treated animals. However, this was found not to be the case since

TABLE II. INCORPORATION OF [$^{14}$C]ACETATE INTO LIPIDS OF LUNG SLICES FROM HAMSTERS TREATED WITH BLEOMYCIN (Bleo) (10 units/kg) INTRATRACHEALLY

| Time after treatment (day) | [$^{14}$C]Acetate into total lipid (dpm/lung/100 g body wt) × $10^5$ | [$^{14}$C]Acetate into lipid as % of uptake[d] |
|---|---|---|
| Control[a] (10)[b] | 5.3 ± 0.4[c] | 12.2 ± 0.7 |
| Bleo-2 (6) | 2.4 ± 0.4 | 4.7 ± 0.7 |
| | P < 0.001[e] | P < 0.001 |
| -4 (6) | 3.3 ± 0.5 | 8.0 ± 1.0 |
| | P < 0.01 | P < 0.01 |
| -7 (6) | 3.3 ± 0.5 | 7.3 ± 1.0 |
| | P < 0.01 | P < 0.01 |
| -14 (5) | 4.0 ± 0.5 | 6.9 ± 0.8 |
| | P < 0.05 | P < 0.001 |

[a] Control values at 2, 4, 7, and 14 days after intratracheal injection of isotonic saline have been pooled since the values were not different.
[b] Numer in parentheses is the number of animals.
[c] Each value represents the mean ± standard error.
[d] See Table I for uptake.
[e] P values are between Control and Bleo groups.

there was no difference in the uptake of [$^{14}$C]acetate by lung slices between the control and bleomycin-treated animals at 4 and 7 days. In fact, a significant increase in the uptake was obtained in the latter case at 2 and 14 days after treatment.

When compared with the control, bleomycin treatment markedly inhibited the incorporation of [$^{14}$C]acetate into total lipids of lung slices for the entire period of the study. Similarly, [$^{14}$C]acetate incorporation into lipid expressed as a percentage of [$^{14}$C]-acetate uptake by the lung slices of bleomycin-treated animals was also significantly less than that of control at all times of the study.

Fractionation of total lipids into various phospholipids and neutral lipid revealed that bleomycin treatment selectively inhibited the synthesis of phosphatidylcholine and neutral lipid. The synthesis of other lipids was variably affected. A marked reduction in incorporation of [$^{14}$C]acetate into neutral lipid and phosphatidylcholine suggests that the [$^{14}$C]acetate was not available in the fatty acid of the diacylglycerol pool for incorporation. One of the reasons why synthesis of these lipids was depressed in the lung slices of bleomycin-treated animals may be the inhibition of fatty acid synthetase complex in these animals. This enzyme is a cytosolic enzyme which is responsible for conversion of

TABLE III. INCORPORATION OF [$^{14}$C]ACETATE INTO VARIOUS LIPID FRACTIONS[a] OF LUNG SLICES FROM HAMSTERS TREATED WITH BLEOMYCIN (10 units/kg) INTRATRACHEALLY

| Time after treatment (day) | SM | PC | PS | PI | PE | NL |
|---|---|---|---|---|---|---|
| | | | (dpm/lung/100 g body wt) | | | |
| Control[b] (10)[c] | 23157 ± 3558[d] | 215941 ± 9652 | 9593 ± 1127 | 9068 ± 664 | 30333 ± 5431 | 191143 ± 15565 |
| Bleomycin | | | | | | |
| 2 (6) | 4713 ± 1409 | 93671 ± 16450 | 4979 ± 1014 | 5802 ± 1112 | 38238 ± 5652 | 79239 ± 10585 |
| | P < 0.001[e] | P < 0.001 | P < 0.01 | P < 0.05 | NS[f] | P < 0.001 |
| 4 (6) | 18286 ± 5408 | 110690 ± 15869 | 9311 ± 1233 | 8220 ± 1110 | 15579 ± 2845 | 137263 ± 17577 |
| | NS | P < 0.001 | NS | NS | P < 0.05 | P < 0.05 |
| 7 (6) | 20692 ± 2504 | 145375 ± 30184 | 7700 ± 1248 | 6432 ± 1403 | 16191 ± 3151 | 145018 ± 22769 |
| | NS | P < 0.05 | NS | NS | P < 0.05 | NS |
| 14 (5) | 31551 ± 8384 | 164307 ± 21194 | 10160 ± 1507 | 9290 ± 1167 | 22673 ± 3717 | 126473 ± 15605 |
| | NS | P < 0.05 | NS | NS | NS | P < 0.02 |

[a] Lipid fractions are abbreviated as follows: SM, sphingomyelin; PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol; PE, phosphatidylethanolamine; NL, neutral lipid.
[b] Control values at 2, 4, 7, and 14 days after intratracheal injection of isotonic saline have been pooled since the values were not different.
[c] Number in parentheses is the number of animals.
[d] Each value is the mean ± standard error.
[e] P values are between the control and bleomycin-treated groups.
[f] NS, not significant.

acetate to fatty acid. It has been demonstrated that 80 to 90% of bleomycin present in the lung is not only associated with the cytosolic portion of the lung parenchyma but also a certain percentage of this compound is covalently linked with the cytosolic protein (27). It is conceivable that decreased incorporation of [$^{14}$C]acetate into neutral lipid and phosphatidylcholine may be due to inhibition of cytosolic fatty acid synthetase complex by bleomycin or its metabolites. Whether incubation of bleomycin with lung slices *in vitro* would have an inhibitory effect on the incorporation of [$^{14}$C]acetate into neutral lipid and phosphatidylcholine is not presently known. However, it should be noted that a pneumotoxin such as 3-methylindole when incubated with goat lung slices inhibited the synthesis of all phospholipids without any effect on neutral lipid (22).

The rate of phosphatidylcholine and sphingomyelin synthesis depends on the availability of choline and activities of three sequential enzymes such as choline kinase, phosphatidylcholine cytidyl transferase, and phosphatidylcholine transferase. It is possible that a marked reduction in the incorporation of [$^{14}$C]acetate into all phospholipids at the early time and phosphatidylcholine at all times in the lung slices of bleomycin-treated animals may be due to a deficiency of choline and/or inhibition of the key enzymes involved in their synthesis. However, it is also possible that bleomycin might have altered metabolism of labeled acetate and its distribution of incorporation into various lipid components of the lung.

Regardless of the mechanism, it should be recognized that phosphatidylcholine is a major component of the lung surfactant. A decrease in the rate of phosphatidylcholine synthesis would lead to a deficiency of lung surfactant and this may constitute the pathogenic basis for bleomycin-induced lung damage as postulated by Burkhardt *et al.* (20) and Gebbers *et al.* (21).

The author acknowledges the skillful help of Mary J. Schiedt.

1. Crooke ST, Bradner WT. Bleomycin: A review. J Med 7:333–428, 1976.
2. Friedman MA. A review of the bleomycin experience in the United States, recent results. Cancer Res **63**:152–168, 1978.
3. Umezawa H. In: Cassady JM, Douros JD, Eds. Anticancer Agents Based on Natural Product Models. New York, Academic Press, pp147–166, 1980.
4. Delena M, Guzzon A, Monfardini S, Bonadonna G. Clinical, radiologic and histopathologic studies on pulmonary toxicity induced by treatment with bleomycin. Cancer Chemother Rep **56**:343–356, 1972.
5. Krous HF, Hamlin WB. Pulmonary toxicity due to bleomycin. Arch Pathol **95**:407–410, 1973.
6. Raisfeld IH. Pulmonary toxicity of bleomycin analogs. Toxicol Appl Pharmacol **56**:326–336, 1980.
7. Snider GL, Celli BR, Goldstein RH, O'Brien JJ, Lucy EC. Chronic interstitial pulmonary fibrosis produced in hamster by endotracheal bleomycin. Amer Rev Respir Dis **177**:289–297, 1978.
8. Starcher BC, Kuhn C, Overton JE. Increased elastin and collagen content in the lungs of hamsters receiving an intratracheal injection of bleomycin. Amer Rev Respir Dis **177**:299–305, 1978.
9. Thrall RS, McCormack JR, McReynolds RM, Ward PA. Bleomycin-induced pulmonary fibrosis in the rat. Amer J Pathol **95**:117–127, 1979.
10. Adamson IYR, Bowden OH. The pathogenesis of bleomycin-induced pulmonary fibrosis in mice. Amer J Pathol **77**:185–198, 1971.
11. Sugiura Y, Kikuchi T. Formation of superoxide and hydroxyl radicals in iron(II)-bleomycin-oxygen system: Electron spin resonance detection by spin trapping. J Antibiotics **31**:1310–1312, 1978.
12. Sausville EA, Peisach J, Horowitz SB. Effect of chelating agents and metal ions on the degradation of DNA by bleomycin. Biochemistry **17**:2740–2754, 1978.
13. Oberley LW, Buettner GR. The production of hydroxyl radical by bleomycin and iron (II). FEBS Lett **97**:47–49, 1979.
14. Ekimoto H, Kuramochi H, Tadahashi K, Matsuda A, Umezawa H. Kinetics of the reduction of bleomycin-Fe(II)$O_2$ complex with DNA. J Antibiotics **33**:426–434, 1980.
15. Sugiura Y, Suzuki T, Kuwahara J, Tanaka H. On the mechanism of hydrogen peroxide, superoxide, and ultraviolet light-induced DNA cleavages of inactive bleomycin-iron(II) complex. Biochem Biophys Res Commun **105**:1511–1518, 1982.
16. Tom WM, Montogomery MR. Bleomycin toxicity: Alterations in oxidative metabolism in lung and liver microsomal fractions. Biochem Pharmacol **29**:3239–3244, 1980.
17. Giri SN, Chen ZL, Younker WR, Schiedt MJ. Effects of intratracheal administration of bleomycin on GSH-shuttle enzymes, catalase, lipid peroxidation and collagen content in the lungs of hamsters. Toxicol Appl Pharmacol **71**:132–141, 1983.
18. Fasske E, Morgenroth K. Experimental bleomycin

lung in mice: A contribution to the pathogenesis of pulmonary fibrosis. Lung **161**:133–146, 1983.
19. Gil J, Reiss OK. Isolation and characterization of lamellar bodies and tubular myelin from rat lung homogenates. J Cell Biol **68**:152–171, 1973.
20. Burkhardt A, Gebbers JO, Holtje WJ. Die bleomycin-lunge systematische pathologische-anatomische untersuchungen an 15 fallen. Disch Med Wochenschr **102**:281–289, 1977.
21. Gebbers JO, Burkhardt A, Holtje WJ. Morphologische lungenveranderungen nach bleomycin-therapie. Verh Disch Ges Pathol **59**:561, 1975.
22. Kirkland JB, Bray TM. The effect of 3-methylindole on the uptake and incorporation of [$^{14}$C]-choline into phospholipids in lung tissue slices. Lipids **19**:709–713, 1984.
23. Skipski VP, Peterson RF, Barklay M. Quantitative analysis of phospholipids by thin layer chromatography. Biochem J **90**:374–378, 1964.
24. Tricke U. Tritosol: A new scintillation cocktail based on Triton X-100. Anal Biochem **63**:555–558, 1975.
25. Karlinsky JB, Goldstein RH. Fibrotic lung diseases —A perspective. J Lab Clin Med **96**:939–942, 1980.
26. Witschi HP. Exploitable biochemical approaches to the evaluation of toxic lung damage. Essays Toxicol **6**:125–191, 1975.
27. Giri SN. Pharmacokinetics, subcellular distribution and covalent binding of [$^{3}$H]-bleomycin in hamsters after intratracheal administration. Exp Mol Pathol **45**:207–220, 1986.

---

Received June 5, 1987. P.S.E.B.M. 1987, Vol. 186.
Accepted July 30, 1987.