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Bleomycin Increases Superoxide Anion Generation by Pig Peripheral Alveolar Macrophages

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SUMMARY

Pulmonary fibrosis is the major toxic effect of bleomycin chemotherapy; however, the molecular mechanisms of the pathological process are unknown. Since alveolar macrophages produce toxic oxygen metabolites and these can damage lung cells, the effect of bleomycin on superoxide anion production was investigated in subpopulations of pig alveolar macrophages. Cells were lavaged from the lung and separated into three subpopulations according to their density. Their capacity to generate superoxide anions increased the more distally they were located. Bleomycin (5.0 milliunits/ml) increased the rate of superoxide production by 75 ± 24% in dense alveolar macrophages located in the lung periphery. Hydrocortisone (10.0 μg/ml) inhibited this superoxide production by 33 ± 10%. Results from this study suggest that an excess production of superoxide anions by alveolar macrophages may be the underlying cause of bleomycin pulmonary toxicity.

Bleomycin is used in the chemotherapy of squamous cell carcinoma, testicular tumors, and lymphomas (1, 2). Pulmonary pneumonitis which may progress to interstitial fibrosis is recognized as the major adverse side effect of bleomycin chemotherapy (3). Despite numerous attempts to delineate the molecular events which may ultimately lead to pulmonary fibrosis (4, 5), no unifying molecular mechanism has been brought forth.

Alveolar macrophages reside in the lung and serve as the initial defenders of the pulmonary system against invading organisms or foreign agents. In response to these agents alveolar macrophages emit a wide variety of products including reactive oxygen species, such as superoxide anions (6). Because reactive oxygen species in excess generate lung injury which may progress to pulmonary fibrosis (7, 8), we decided to examine the effect of bleomycin on superoxide anion generation by pig alveolar macrophages. Moreover, following bleomycin exposure, alveolar macrophages have been shown to secrete chemotactic factor (9, 10) and collagenase (11). However, alveolar macrophages are not a homogeneous population of cells. In fact, subpopulations exist which possess different morphological and functional properties (12–15).

In order to examine alveolar macrophages from various regions of the lung, the cells were collected after sequentially lavaging the lung beginning with the trachea and ending with the lung fully expanded. The cells obtained were separated into subpopulations according to their densities. Results from this study indicate that bleomycin increases substantially the rate of superoxide production by alveolar macrophages and that this enhancement can be partially inhibited by hydrocortisone exposure.

Materials and Methods

Animals. Male and female pigs from 1 to 3 weeks old were obtained from a local commercial hog farm. It has been previously shown that alveolar macrophages from 7-day-old pigs are not different from adult pig alveolar macrophages (16).

Isolation of alveolar macrophages. Pigs were anesthetized with sodium pentobarbital and alveolar macrophages were obtained by lung lavage as previously described by Zeidler and Kim (16). The lavage medium consisted of 145 mM NaCl, 10 mM glucose, and 10 mM sodium phosphate buffer at pH 7.4. Lungs were inflated to a given volume by initially ascertaining the volume of air needed to maximally expand the lung. After deflation, the lungs were then expanded with a known volume of medium to lavage a specific region of the lung. The trachea was filled with several ml of ice-cold lavage medium and this was passed back and forth through the trachea 15 times. These cells were designated cells from the trachea. Next the lung was filled with medium until it was one-fourth expanded. This medium was passed back and forth through the lung 15 times. These cells are those designated from one-fourth expanded lung. A similar procedure was followed for half and fully expanded lungs, except that fresh lavage medium was used after an initial wash out. It should be noted that when pig lungs are lavaged 5 times, little or no further cells are obtained upon subsequent lung washings. Therefore, 15 lavages were more than adequate to remove cells from a particular region of the lung, before proceeding to the next area to be lavaged.

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ABBREVIATION: PMA, phorbol myristate acetate.

Density separation of macrophage subpopulations. Alveolar macrophages were separated into subpopulations according to their densities on continuous density gradients of Percoll (Pharmacia, Piscataway, NJ) as described by Dauber *et al.* (15). Gradients were self-generated by centrifuging 30 ml of Percoll in 50-ml tubes at 18,000 × *g* at 5° for 90 min. Macrophages (3×10^7) were layered onto gradients and centrifuged at 400 × *g* for 20 min. The alveolar macrophages were separated into three distinct bands on these gradients. Cells from each band were collected and washed two times by alternate centrifugation and resuspension in the lavage medium.

Area ratio determination. The cytoplasm/nucleus ratio was determined by measuring these areas located in cells stained on glass slides. The cells were projected onto a screen utilizing a projector (Zeiss, model 1106, Hitscfel Optical Instruments, St. Louis, MO), and the outlines of specific areas were drawn on paper. These areas were cut out and weighed. Ratios of areas were then calculated.

Cell volume. Cell volume was measured as previously described (16). Volumes were determined by dispersing macrophages in Isoton at room temperature. Cells were passed through an aperture with a 50-m diameter opening and 60-m length to prevent distortions in volume measurements. Cells were analyzed by utilizing a Coulter Channelyzer model C-1000 interfaced with the model ZBI Coulter Counter (Coulter Electronics).

Superoxide anion production. The production of superoxide anions was measured by the superoxide dismutase inhibitable reduction of cytochrome *c* following the procedure of Tritsch and Niswander (17). Cells (5×10^6) were suspended in a balanced salt solution consisting of 145 mM NaCl, 5 mN KCl, 0.5 mM $CaCl_2$, 0.1 mM $MgSO_4$, 10 mM glucose, and 10 mM sodium phosphate buffer, pH 7.4, at 38°. To this was added 0.5 ml of 1.2 mM cytochrome *c* (type III, Sigma Chemical Co.). The reaction was initiated by the addition of 0.5 μg/ml of PMA. Samples of cells and incubation medium were removed at 0, 7, and 15 min and centrifuged at 8000 × *g* in an Eppendorf microcentrifuge for 5 sec.

The optical density of the supernatant fluid was determined at 550 nm. Superoxide produced was calculated from the concentration of cytochrome *c* reduced by the extinction coefficient of $E = 2 \times 10^4$ $M^{-1}cm^{-1}$. Superoxide dismutase (100 μg/ml) was added to a companion mixture to account for superoxide dismutase-inhibitable superoxide production. Superoxide-inhibitable superoxide production is calculated from the difference in superoxide anions produced by cells exposed to superoxide dismutase and companion cells not exposed to superoxide dismutase. Bleomycin was added to the incubation medium in final concentrations of 1.0, 5.0, 10.0, and 20.0 milliunits/ml. The alveolar macrophages were preincubated for 1.0 min with bleomycin prior to the stimulation of superoxide anion production. Cytochrome *c*, PMA, and superoxide dismutase were obtained from Sigma Chemical Co., St. Louis, MO. Bleomycin sulfate was supplied by Bristol Laboratories, Syracuse, NY.

Results

When the entire lung was lavaged at once, the pig alveolar macrophages were separated into three subpopulations according to density (Fig 1). The density of band 1 was 1.032 ± 0.003. The density of bands 2 and 3 were 1.045 ± 0.002 and 1.067 ± 0.003, respectively. The typical cell volumes of bands 1 and 2 were 1700 ± 100 and 1505 ± 176 μm^3. The volume of band 3 cells was 1203 ± 157 μm^3.

When lung was lavaged sequentially beginning with the wash out of the trachea only and proceeding to the wash out of the one-fourth, half, and fully expanded lung, the cells were also separated into three subpopulations with the same densities as before. However, their cell volumes were quite different. This is demonstrated in Fig. 2. The cells located in band 1 had volumes which decreased as the cells located in the lung periphery were obtained by lavage. Cells located in band 2 also

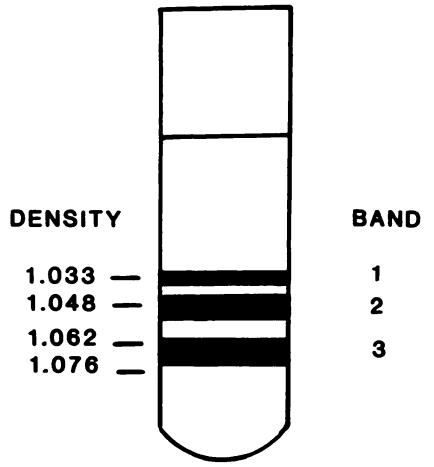

Fig. 1. Density separation of pig alveolar macrophage. Three subpopulations were isolated when lavaged cells were centrifuged on density gradients of Percoll. Density is mg/ml. Cells from the whole lung were examined.

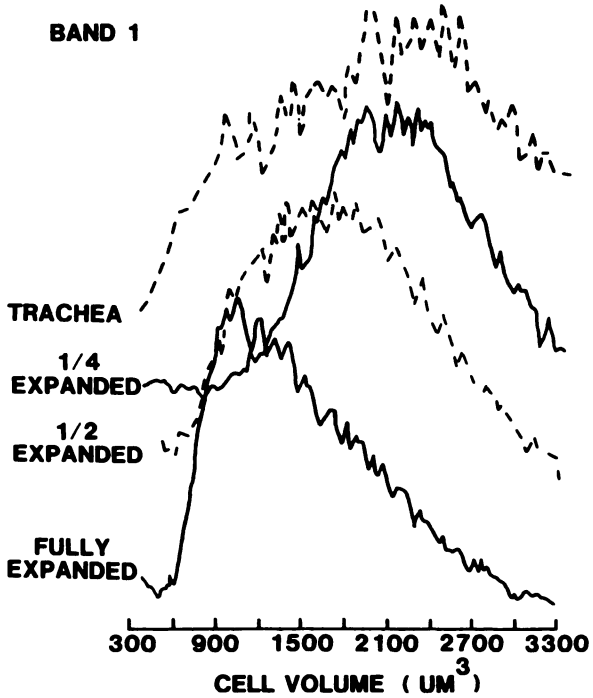

Fig. 2. Cell volumes of band 1 cells. Band 1 cells were collected from the density gradients of cells lavaged from the various regions of the lung. A decrease in cell volume is noted when cells located in the outer regions of the lung are examined.

exhibited a decrease in cell volume (Fig. 3) as they were lavaged from the lung. No cells from band 3 were found in the trachea (Fig. 4). However, band 3 cells lavaged from the remainder of the lung did exhibit a decrease in cell volume as they were dislodged from their resting sites in the lung periphery.

Not only did cell volume vary as the cells were sequentially washed from the lung, but the number of cells also increased. This may be expected since a larger surface area of the lung is being lavaged as the lung expands during the lavage procedure. These results are reported in Table 1.

The cells with various densities and cells recovered from various regions of the lung also differed morphologically. The ratios of nucleus area to cytoplasma area were quite different. Cells with a ratio less than 1 would contain more cytoplasmic

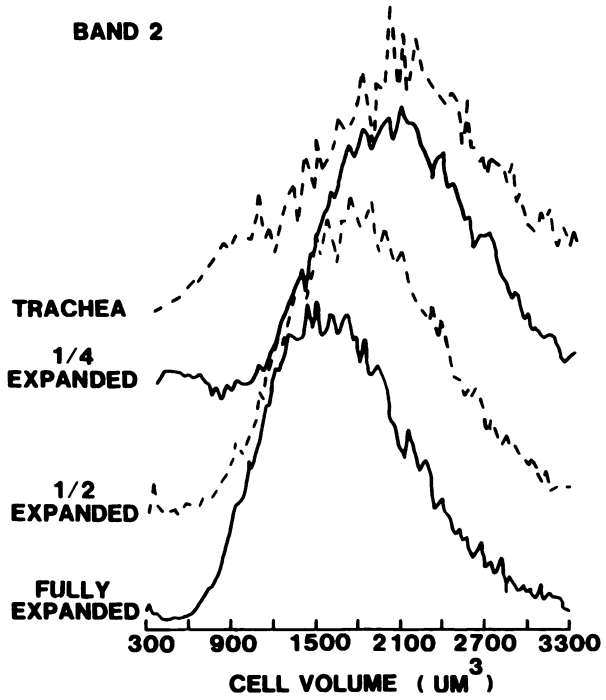

Fig. 3. Cell volumes of band 2 cells. These macrophages were isolated from various lung regions and separated from other subpopulations by their density. A decrease in cell volume is manifested as cells from the more peripheral regions of the lung are measured.

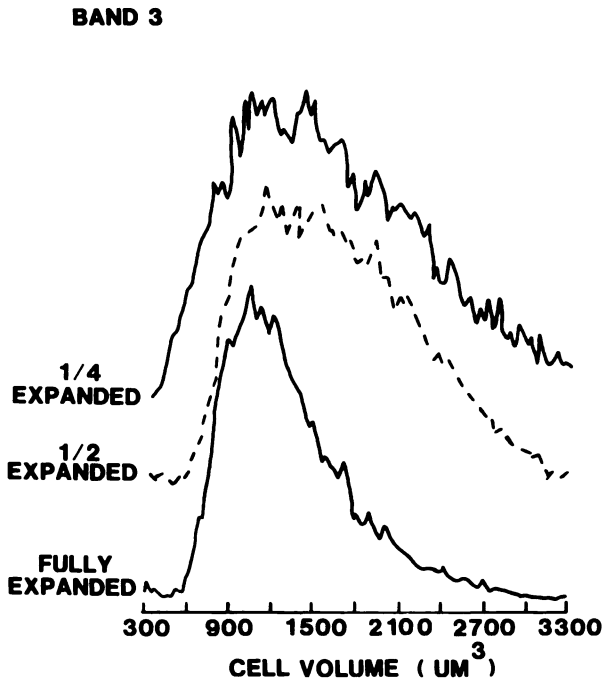

Fig. 4. Cell volumes of band 3 cells. Volumes were measured from lavaged cells collected in various portions of the lung and separated according to density. No band 3 cells were found in the trachea. Cellular volumes decreased in macrophages located in the lower regions of the lung.

area than nuclear area. Approximately 55% of the cells lavaged from the trachea were identified as cells having ratios less than 1 (Table 1). As cells from the peripheral regions of the lung were lavaged free and recovered, the percentage of cells with ratios less than 1 decreased. It can also be noted that cells from the more peripheral regions of the lung had smaller volumes, even though their densities were the same as those of cells located in the upper airways.

TABLE 1
Characteristics of lavaged bronchoalveolar cells[a]

	Band	Cell number	Volume	Nucleus area/cytoplasm area ratio < 1
		1×10^6 cells	μm^3	%
Trachea	1	2.5 ± 2.3[b]	1980 ± 396	50 ± 8
	2	17.1 ± 6.6	1920 ± 214	60 ± 4
	3			
¼ Expanded lung	1	5.9 ± 1.2	1920 ± 401	50 ± 7
	2	14.2 ± 5.6	1800 ± 384	55 ± 7
	3	19.5 ± 17.8	1320 ± 238	80 ± 9
½ Expanded lung	1	7.7 ± 3.6	1590 ± 415	50 ± 10
	2	39.1 ± 14.6	1635 ± 318	40 ± 3
	3	34.9 ± 15.6	1365 ± 385	50 ± 5
Fully expanded lung	1	16.3 ± 9.3	1290 ± 235	35 ± 4
	2	56.6 ± 16.3	1425 ± 310	40 ± 6
	3	56.0 ± 22.0	1125 ± 205	16 ± 3

[a] $n = 7$.
[b] Values are means ± standard deviations.

TABLE 2
Superoxide anion production by bronchoalveolar cells[a]

	Band	Rate of superoxide production
		nmol/1 × 10^6 cells × min
Trachea	1	0.39 ± 0.19[b]
	2	0.25 ± 0.12
	3	
¼ Expanded lung	1	0.12 ± 0.06
	2	0.32 ± 0.15
	3	0.34 ± 0.16
½ Expanded lung	1	0.59 ± 0.27
	2	0.38 ± 0.16
	3	0.67 ± 0.22
Fully expanded lung	1	0.62 ± 0.30
	2	0.44 ± 0.19
	3	0.70 ± 0.30

[a] $n = 4$.
[b] Values are means ± standard deviations.

The capacity of the alveolar macrophages to generate superoxide anions also varied considerably (Table 2). Cells washed from the upper airways produced less superoxide anions than cells washed from the lower airways.

Band 3 cells washed from the fully expanded lung represent alveolar cells from the peripheral lung. Since this is the site of bleomycin toxicity, these cells were used to investigate the ability of bleomycin to enhance the generation of superoxide anions. As shown in Fig. 5, bleomycin increased PMA-initiated superoxide production; however, no further enhancement occurred beyond 5 milliunits/ml. The average increase in production at 5 milliunits/ml was 75 ± 24% ($n = 6$). Since therapeutic concentration ranges in humans vary between 1.0 and 10 milliunits/ml, the concentrations used in Fig. 5 have *in vivo* significance. Bleomycin without PMA did not produce detectable superoxide formation.

In order to ameliorate pulmonary inflammations, hydrocortisone is often used as a therapeutic agent. Therefore, the effect of hydrocortisone on bleomycin-enhanced superoxide production by macrophages was examined. Alveolar macrophages were

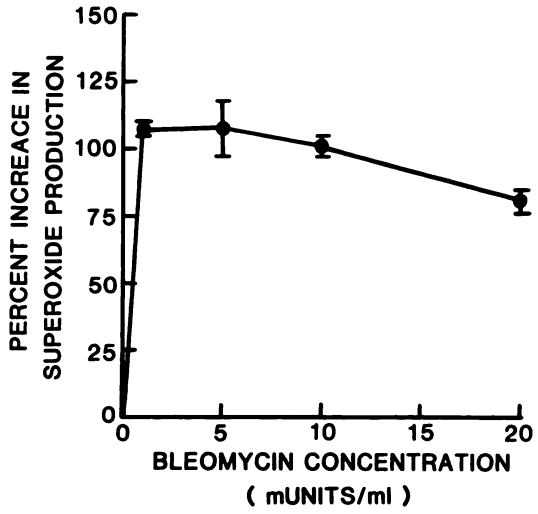

Fig. 5. Bleomycin enhancement of superoxide anion production by band 3 alveolar macrophages. Typical values ± range from two pigs are shown. Superoxide anion production is initiated by the addition of 0.5 μg/ml of PMA.

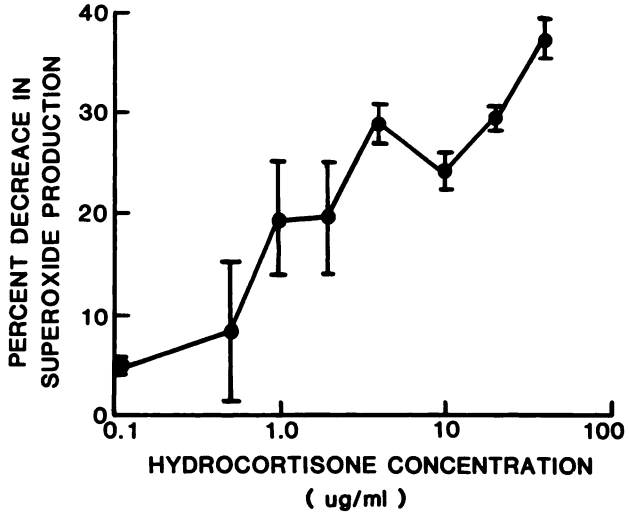

Fig. 6. Hydrocortisone inhibition of superoxide production in the presence of bleomycin. Cells are preincubated 5 min with various concentrations of hydrocortisone and 5 milliunits/ml of bleomycin. An increased inhibition occurs as the external concentration of hydrocortisone increases. Typical values ± range from two pigs are shown.

preincubated 5.0 min with various concentrations of hydrocortisone. Cells were then stimulated with PMA. Superoxide production in the presence of hydrocortisone is shown in Fig. 6. The magnitude of inhibition increased as the medium concentration of hydrocortisone increased from 0.1 to 40 μg/ml. At a therapeutic concentration of 10 μg/ml, an inhibition of 33 ± 10% ($n = 5$) occurred.

Discussion

Alveolar macrophages isolated from pig lungs consist of cells with varying cell volumes, densities, and capacities to generate superoxide anions. This is consistent with results reported by Dauber *et al.* (15), Shellito and Kaltreider (13), and Holian *et al.* (18) in other species. However, their studies describe results from cells lavaged from the whole lung. Our results extend these observations by reporting that cells from various regions of the lung also differ markedly. Most notable are the decrease in cell volume and increase in rates of superoxide production of cells lavaged from more peripheral regions of the lung. Alveolar macrophages that are generating superoxide anions produce more in the presence of bleomycin.

Since bleomycin by itself did not stimulate the alveolar macrophages to produce superoxide anions, bleomycin may interact with components of this process, once the cells are stimulated. Superoxide anions are generated by a membrane-bound NADPH oxidase which participates in an electron transport chain including a flavoprotein moiety and an iron-associated cytochrome *b* (19). Bleomycin may combine with the macrophage membrane and enhance this process or may form an iron-oxygen complex and generate superoxide anions itself. An iron-bleomycin-oxygen complex has been implicated in tumor cell DNA damage (20).

The enhanced superoxide anion generation caused by bleomycin may also be a result of bleomycin interacting with protein kinase c. PMA binds to and activates membrane-bound protein kinase c, which includes the phosphorylation of membrane protein (21). Bleomycin may increase superoxide generation at this step in the process by effecting the binding of PMA to protein kinase c. This in turn could lead to greater membrane protein prosphorylation and superoxide anion generation. These possible mechanisms are areas for future studies.

Corticosteroids are used therapeutically in the treatment of selected pulmonary inflammations (22). Among other things, they stabilize lysosomal membranes, inhibit complement-mediated damage, and inhibit leukocyte adherence. In addition, hydrocortisone inhibits the release of secretions of monocytes (23). In this study, we have shown that hydrocortisone significantly decreases the rate of superoxide anion generated by PMA-stimulated alveolar macrophages. This effect may explain in part the results of White and Stover (24) who observed a decrease in the severe symptoms of patients with bleomycin pulmonary toxicity who received corticosteroid therapy.

Results from this study suggest that bleomycin pulmonary toxicity may be the result of an excess production of superoxide anions and oxygen-reactive species by alveolar macrophages, which are stimulated by natural agents in the presence of bleomycin. These oxygen species may overwhelm the natural defenses of the lung which consist of superoxide dismutase, catalase, and glutathione. As a result, lung parenchymal cell damage occurs. Pulmonary fibrosis may eventually result from this bleomycin toxicity.

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