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Bleomycin Binding Sites on Alveolar Macrophages

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Previous work has demonstrated that bleomycin can directly stimulate alveolar macrophage secretion of fibroblast growth factors and monocyte chemotactic factors. In this study, rat alveolar macrophages obtained by bronchoalveolar lavage were examined for the presence of bleomycin binding sites, which might mediate this response. The results indicated that alveolar macrophages have specific, saturable, and reversible binding sites. Both high- and low-affinity binding sites were found; each macrophage possessed 6.7×10^4 high-affinity sites, with a K_d of 528 nM, and 2.2×10^6 low-affinity sites, with a K_d of 65 μM. The K_d of the high-affinity sites corresponds closely to the ED_{50} obtained from dose-response curves of the bleomycin-stimulated secretion of both fibroblast growth and monocyte chemotactic factors, suggesting that bleomycin stimulation of alveolar macrophage function responses may be mediated by bleomycin interaction with these sites.

Key words: pulmonary fibrosis, lung, fibroblast growth factors, monocyte chemotactic factors

INTRODUCTION

Pulmonary fibrosis induced by the administration of bleomycin in a number of rodent species has been established as an animal model of the human disease [11]. Alveolar macrophages isolated from animals with bleomycin-induced fibrosis secrete cytokines [6,15], fibroblast growth [2,4,8,11] and inhibitory factors [3], and leukocyte chemotactic factors [5,7], all of which are thought to play important roles in mediating the fibrotic reaction of the lungs to this drug. It has also recently been demonstrated that similar results can be obtained by the in vitro stimulation with bleomycin of alveolar macrophages from normal animals [4–6,15].

The experiments presented here were undertaken to determine if alveolar macrophages possess specific binding sites for bleomycin. Bleomycin internalization by both tumor and normal cells has been established directly by studies of drug uptake [9,13] and indirectly by the demonstration of chromosomal damage following the incubation of cells with this drug [1,12,17]. However, evidence that this internalization is a receptor-mediated event has been lacking. In support of this hypothesis, it has been demonstrated that the lethal effects of bleomycin on Chinese hamster ovary (CHO) cells can be ablated by pretreatment of cells with trypsin, implying the presence of an intermediary membrane protein(s) involved in the interaction of this drug and CHO cells [1]. As described here, the finding of bleomycin binding sites on alveolar macrophages not only suggests a mechanism by which these cells may be stimulated to secrete monokines in bleomycin-induced pulmonary fibrosis but may also indicate a mode of action of this drug that is in addition to its well known cytotoxic effects, mediated by DNA strand breakage [1,11,12,17].

MATERIALS AND METHODS

Materials

Bleomycin (Blenoxane) was a gift from Bristol Laboratories (Bristol-Myers Company, Evansville, IN). [S-methyl-^{3}H]-bleomycin A_2, copper form (63-74 Ci/mmol), was purchased from New England Nuclear (Boston, MA). High-pressure liguid chromatography (HPLC)-grade CH_3CN and CF_3COH were obtained from Pierce (Rockford, IL). Trypan blue and Wright's stain solution were from Sigma Chemical Co. (St. Louis, MO). All other chemicals used to prepare buffers were of reagent grade or better and were purchased from Fisher Scientific Co. (Livonia, MI).

Methods

Purification of bleomycin A_2. Blenoxane is a mixture of bleomycin A_2 and B_2 [15]; for use in binding assays, bleomycin A_2 was purified using reverse-phase HPLC with a Waters Associates 510 chromatograph system (Waters, Div. of Millipore, Milford, MA). The column was an RP 318 (Biorad, Richmond, CA), with 3

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μm particle size and measuring 4.6 × 250 mm. Elution was accomplished isocratically at 22°C with 89% solvent A (0.1% CF_3CO_2H in H_2O) was 11% solvent B (0.1% CF_3CO_2H in CH_3CN) and a flow rate of 1.0 ml/min. Under these conditions, as detected by absorbance at 214 nm, bleomycin A_2 and B_2 eluted at 20 and 31 min, respectively. Material eluting at 20 min from ten column runs was collected using a fraction collector and pooled, dried using vacuum centrifugation (Speed Vac, Savant Instruments Inc., Hicksville, NY), and resuspended in 95% ethanol, and stored at − 20°C. Using this chromatography method, the purity of each commercially purchased batch of [^{3}H]bleomycin A_2 was also checked before use and was found to consist of ≥97% bleomycin A_2.

Alveolar macrophage isolation. Macrophages were obtained from male-specific pathogen-free Fisher 344 rats weighing 150–200 g (Charles River, Portage, MI) exactly as reported previously [4]. Briefly, rats were killed with an overdose of ketamine, and lungs were perfused with 50 ml of sterile phosphate-buffered saline (PBS) containing 1 mM EDTA and 0.1% glucose to remove blood. Lungs were then lavaged with ten, 10 ml washes of this sterile PBS solution. Following isolation, macrophages were resuspended in Hank's balanced salt solution (HBSS) supplemented with 1 mM $CaCl_2$, 1 mM $MgSO_4$, and 0.1% (wt/v) glucose, pH 7.2, and placed in an ice water bath. These cells were ≥98% macrophages by analysis of cytocentrifuge (Shandon Instruments, Pittsburgh, PA) preparations stained with Wright's stain. Viability in all cases was >95% as assessed by exclusion of trypan blue.

Bleomycin Binding Assays

Both radioactive and unlabeled bleomycin A_2 were dried using vacuum centrifugation to remove ethanol in stock solutions, then resuspended in supplemented HBSS. Polypropylene microfuge tubes (Sarstedt, Princeton, NJ) were siliconized with Prosil-28 (PCR Inc., Gainesville, FL) 24 hr prior to use. Cells, buffers, and bleomycin were chilled to 4°C before use, and all incubations with ligand were done at 4°C. Each tube contained 1×10^6 macrophages and the indicated concentration of [^{3}H]bleomycin. Nonspecific binding was determined as dpm bound in the presence of a 100-fold concentration of unlabeled bleomycin A_2. For kinetic studies, incubations were at 4°C for the times indicated; for all other experiments, the incubations were for 60 min. All incubations were stopped by rapid filtration and washing of cells with ice-cold HBSS using a Brandel cell harvester (Gaithersburg, MD). To reduce nonspecific binding of the radiolabeled ligand, GF/B filters used in harvesting cells were presoaked in 1% (wt/v) powdered milk (Carnation) in HBSS just prior to use. All tubes were washed eight times with 1.5 ml of ice-cold HBSS. Filters were dried and placed into scintillation vials and counted for radioactivity after the addition of scintillant (Ready Protein; Beckmann Instruments, Fullerton, CA).

Analysis of radioactivity bound to macrophages. Four million to six million macrophages per tube were incubated with 169 nM (0.25 μg/ml) of [^{3}H]bleomycin for 1 hour at 4°C as described above. Following the incubation, tubes were centrifuged (1,500 rpm) for 5 min at 4°C, and the supernatant was discarded. Cell pellets were then resuspended in 1 ml ice-cold HBSS and washed thrice by centrifugation; after the last wash, 50 μl of 6.77 mM (10 mg/ml) nonradioactive bleomycin A_2 was added followed by 500 μl of 5% trichloroacetic acid (TCA) (4°C) and vortexed. After 15 min on ice, TCA-precipitable protein was pelleted by centrifugation. The resultant supernatants were filtered (0.2 μm Acro LC13 HPLC filters; Gelman Sciences, Ann Arbor, MI) prior to analysis, using the HPLC method described above. Peak absorbance of unlabeled bleomycin A_2 added to the samples was compared to the elution time of radioactivity in the fractions. To ensure that [^{3}H]bleomycin was precipitated and unaltered by TCA, [^{3}H] bleomycin was incubated in the absence of macrophages then treated with TCA as described for cell samples. Binding data were analyzed using the LIGAND computer program [10] to determine the number and affinity of the binding sites.

RESULTS

When alveolar macrophages were incubated with concentrations of 20–474 nM (0.3–0.7 μg/ml) of [^{3}H]-bleomycin, total binding increased with the concentration of bleomycin present, saturating between 200 and 474 nM (Fig. 1). Nonspecific binding, in the presence of a 100-fold excess unlabeled bleomycin, increased linearly with increasing concentrations of radioactive ligand. The specific binding, represented as the total minus the nonspecific radioactivity bound, increased in linear fashion from 20 to 169 nM and did not increase further at doses between 169 and 474 nM. Alveolar macrophages thus appear to have specific and saturable membrane binding sites for bleomycin A_2.

To determine the kinetics of bleomycin binding to these sites, macrophages were incubated with a saturating concentration of [^{3}H]bleomycin (338 nM) in the presence or absence of 3.38 μM of unlabeled bleomycin for 2–60 min. Binding of the ligand to macrophages was rapid, approaching maximal levels after 10 min (Fig. 2). Although there was some increase in specifically bound radioactivity from 10 to 80 min, this difference was found not to be statistically different in any of the three experiments performed.

The reversibility of bleomycin binding to macrophages was examined by incubating cells for 60 min with

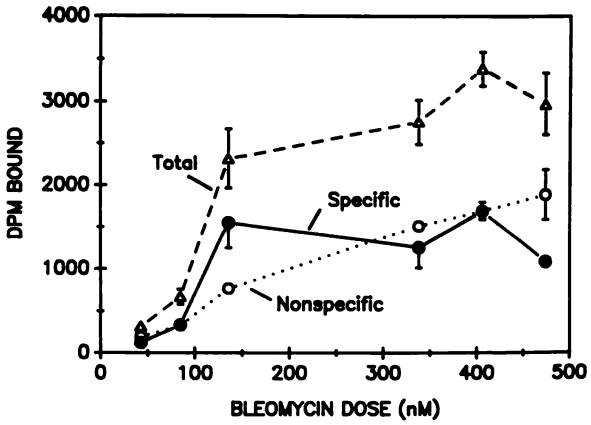

Fig. 1. Dose response of bleomycin binding. Macrophages were incubated at 4°C for 60 min with the indicated concentration of [^{3}H]bleomycin A_2 in the presence (open circles) or absence (triangles) of a 100-fold excess unlabeled bleomycin A_2. Specific binding (solid circles) was determined by subtracting nonspecific binding from total binding. Data represent the mean ± SE (of triplicate data points) of a representative experiment from a total of 10 such experiments.

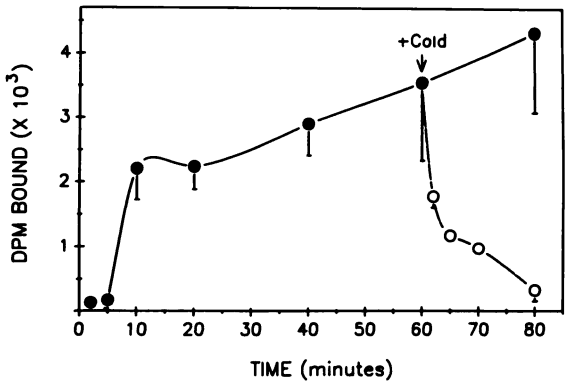

Fig. 2. Kinetics of bleomycin binding. Macrophages were incubated at 4°C for the indicated times with 338 nM [^{3}H]bleomycin A_2. Specific binding was determined as described in the legend to Figure 1. To examine reversibility, one set of tubes was incubated for 60 min with [^{3}H]bleomycin A_2 prior to the addition of unlabeled bleomycin (arrow); specific binding remaining at 2, 5, 10, and 20 min after this addition was then determined (broken line). Data represent the mean ± SE (of triplicate data points) from a total of three such experiments.

[^{3}H] bleomycin, then adding a 100-fold excess unlabeled ligand for an additional time of 2–20 min (Fig.2, broken line). [^{3}H]bleomycin was rapidly displaced from binding sites, with a reduction in specifically bound radioactivity evident as early as 2 min after addition of the unlabeled drug; this displacement was essentially complete in 20 min.

The characteristics of these binding sites were analyzed by the ability of increasing doses of cold ligand to displace the binding of radioactive ligand. This was

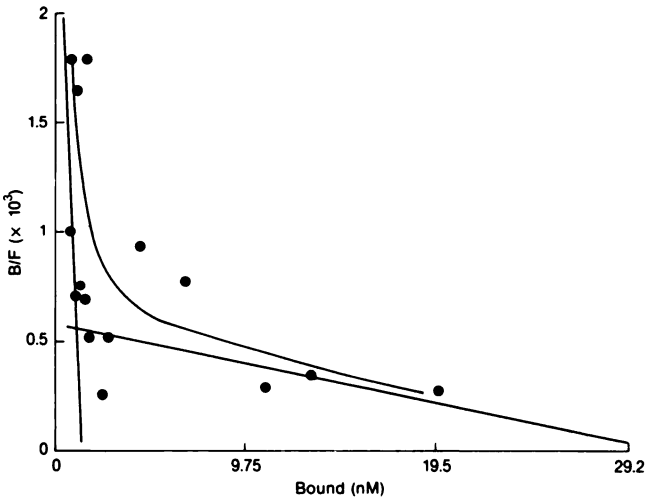

Fig. 3. Scatchard plot of bleomycin binding. Macrophages were incubated at 4°C for 60 min with the indicated total concentration of bleomycin, a constant 271 nM of which was [^{3}H]bleomycin A_2. Bound radioactivity was converted to nmoles; data are the means of duplicate data points from four experiments.

accomplished by incubating alveolar macrophages with a constant concentration of 271 nM [^{3}H]bleomycin and increasing concentrations of cold ligand. Analysis of the data from four such experiments by LIGAND revealed the presence of both high- and low-affinity binding sites (Fig. 3). Each macrophage possessed an average of 6.7×10^4 sites, with an apparent K_d of 528 nM, and an average of 2.2×10^6 low-affinity sites, with an apparent K_d of 65 μM. Although low-affinity binding sites were evident in all such experiments, it still remains a possibility that these sites represented nonspecific binding to the plasma membrane.

Finally, HPLC analysis of radioactive bleomycin bound to macrophages under the binding assay conditions described (60 min, 4°C) verified that [^{3}H]bleomycin A^2 was not metabolized or degraded during the assay period (Fig. 4A). Ninety percent of the radioactivity recovered from macrophages incubated with [^{3}H]bleomycin coincided with peak absorbance of nonlabeled bleomycin A_2. In parallel controls of [^{3}H]bleomycin incubated in the absence of macrophages, 91% of the radioactivity recovered eluted along with the unlabeled ligand (Fig. 4B).

DISCUSSION

The results of this study revealed that bleomycin could bind to alveolar macrophages in a rapid, specific, and saturable manner. Since all experiments were performed at 4°C, it is likely that cell-associated radioactivity reflected bleomycin bound to membrane sites on the cell surface rather than inside the cell. This conclusion is

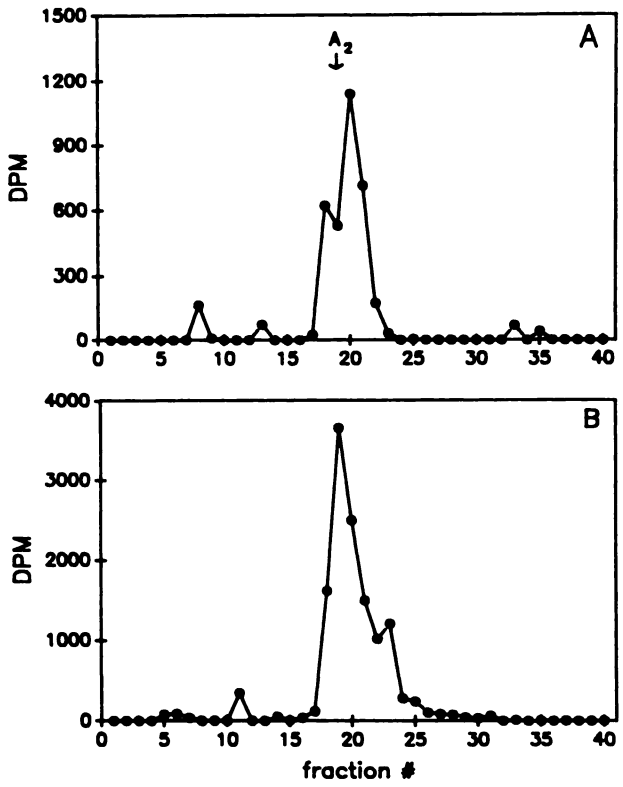

Fig. 4. HPLC analysis of radioactivity bound to macrophages. [^{3}H]bleomycin was incubated with (A) or without (B) macrophages for 60 min at 4°C, after which cells were washed to remove unbound ligand. Unlabeled bleomycin was added as a tracer and to enhance dissociation of ligand from cells. [^{3}H]bleomycin recovered in supernatants following TCA precipitation was isolated by HPLC as decribed in Methods. Radioactivity (dpm) and absorbance were determined for each fraction. Arrow indicates absorbance (214 nm) peak of unlabeled bleomycin A_2.

supported by the rapid reversibility of binding (Fig. 2) and by studies with Ehlrich cells [9], which have shown that the association of this drug with cells was irreversible once it was internalized. Furthermore, studies on the effects of temperature on the endocytosis of ligands by alveolar macrophages [16] have indicated that internalization is negligible at 4°C. The findings presented here are therefore compatible with the existence of bleomycin binding sites on alveolar macrophages.

This demonstration of bleomycin binding sites on macrophages is significant in that the K_d of the high-affinity sites (528 nM) is within the concentration range of the doses of bleomycin which evoke a biological response. Previous work in this laboratory has shown that alveolar macrophages obtained from normal rats incubated with 6.77–670 nM bleomycin in vitro secreted both monocyte chemotactic factor and macrophage-derived growth factor (MDGF) [4,5]. Jordana et al. [6] have likewise demonstrated enhanced secretion of interleukin-1 by alveolar macrophages incubated with 339 nM bleomycin. Time course experiments have also shown that the kinetics of [^{3}H]bleomycin binding to macrophages (Fig. 2) are in accordance with the time course of the secretion of both MDGF and monocyte chemotactic factor, which were detectable as early as 30 min following stimulation with bleomycin in vitro [4] (also, unpublished observations). The differences in the optimal concentrations of bleomycin necessary for measuring binding and that needed to elicit a biological response may be due to the differences in the incubation temperatures used; binding assays were carried out at 4°C whereas experiments on the effects of bleomycin on the secretion of MDGF and chemotactic activity [4,5] were carried out at 37°C.

Since bleomycin is a glycopeptide product of *Streptomyces verticullus* [16], it is unclear why alveolar macrophages would have specific binding sites for this drug. Binding sites for such glycopeptides may serve in a protective capacity similar to that suggested for the receptors for bacterial peptides [14] and lipopolysaccharide [18], allowing alveolar macrophages to respond to airborne mold products. It also seems likely that the bleomycin binding sites represent membrane sites with other functions that can also bind to bleomycin; these binding sites may be nonspecific, reacting with a range of glycosalated compounds [16], or specific receptors for some endogenous mediator(s) capable of regulating macrophage function in both homeostasis and disease [11]. The demonstration of these binding sites provides important support for the existence of novel cellular and tissue effects of this drug in addition to its well-known effects on cytotoxicity and DNA strand scission [17].

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