Enhancement of Bactericidal Capacity of Alveolar Macrophages by Human Alveolar Lining Material

JOHN A. JUERS, ROBERT M. ROGERS, JOEL B. MCCURDY, and WILLIAM W. COOK

From the Pulmonary Disease Section, Department of Medicine, The University of Oklahoma Health Sciences Center, and the Veterans Administration Hospital, Oklahoma City, Oklahoma 73190

ABSTRACT In vivo studies have shown a major role for the alveolar macrophage in the killing of inhaled bacteria. This contrasted with earlier work which showed a preservation of phagocytic properties but a loss of bactericidal capacity when alveolar macrophages were studied in vitro. Recently, alveolar lining material (ALM) from rats has been shown to enhance the in vitro bactericidal capacity of alveolar macrophages from homologous animals against *Staphylococcus aureus*. Utilizing a similar system, we have confirmed that rat alveolar macrophages do not kill *S. aureus* in vitro unless the bacteria have been incubated with rat ALM (R-ALM) before phagocytosis. In addition, human ALM (H-ALM) from 7 of 11 patients assayed showed an enhancement of bactericidal capacity by rat alveolar macrophages which was not significantly different from the results utilizing R-ALM. H-ALM from the other four patients gave results which differed significantly from results with H-ALM from the first seven patients and R-ALM ($P < 0.001$). Preliminary results suggest that the factor enhancing the bactericidal capacity of rat alveolar macrophages is present in the lipid fraction of the ALM. Further characterization of the ALM is warranted in an effort to explain the enhancement of the bactericidal capacity of alveolar macrophages by most, but not all, H-ALM tested.

INTRODUCTION

Experimental models have suggested that a variety of factors, such as pulmonary edema, immunosuppressive agents, oxygen toxicity, and physical stress, impair host defenses against bacterial infection of the lungs (1–6). This may be a result of impaired alveolar macrophage bactericidal activity (7, 8), as well as enhanced bacterial replication in the lung (9). In vitro studies suggested that normal alveolar macrophages (10, 11), as well as alveolar macrophages from physically-stressed animals (4, 5), demonstrate normal phagocytic ability, but fail to kill ingested bacteria. Intracellular killing of *Staphylococcus aureus* (12) and *Pseudomonas aeruginosa* (13) by rat alveolar macrophages in vitro has been shown to be markedly enhanced by the cellular fraction of lung washings from homologous animals. With the in vitro system of LaForce et al. (12), we confirmed the enhancement by rat alveolar lining material (R-ALM)[1] of the bactericidal capacity of rat alveolar macrophages against *S. aureus*. We have also demonstrated that human alveolar lining material (H-ALM) can be utilized in the in vitro assay and that most samples facilitated heterologous (rat) alveolar macrophage bactericidal function. Preliminary observations suggest that the lipid fraction of H- and R-ALM may be responsible for the observed phenomenon.

METHODS

Animals. Male Wistar rats (Charles River Breeding Laboratory, Wilmington, Mass.), weighing 250–500 g were used in all experiments. Animals were housed at least 3 wk before experimentation and were fed and watered ad libitum.

Bacteria. S. aureus (ATCC 6538P) was used in all studies. The bacteria were maintained on blood agar plates at room temperature. 16-h cultures, grown in tryptic soy broth, were used in each experiment.

Harvest of alveolar macrophages. Rats were anesthetized with sodium pentobarbital, 50 mg/kg, intraperitoneally. Blood was aspirated from the abdominal aorta as a source of fresh serum; animals then were exsanguinated by aortic transection. The trachea was cannulated with sterile poly-

Received for publication 15 September 1975 and in revised form 24 February 1976.

[1] *Abbreviations used in this paper:* ALF, alveolar lipid fraction; ALM, alveolar lining material; H-ALM, human alveolar lining material; HBSS, Hank's balanced salt solution; R-ALM, rat alveolar lining material.

ethylene tubing, and the lungs were lavaged with five 10-ml washes using heparinized, normal saline (10 U/ml, 24°C). Lavage effluents, collected on ice, were centrifuged at 500 *g* for 5 min. The cell buttons were immediately resuspended in Hank's balanced salt solution (HBSS) and stored on ice. Sediments with visible erythrocyte contamination were discarded. Total cell counts were performed with a hemocytometer, viability determined by the methylene blue reduction technique, and differential cell counts performed on smears stained with Wright's stain. The cell suspensions were adjusted with a volume of HBSS to yield a concentration of approximately 1.5×10^6 cells/ml.

Alveolar lining material (ALM). 100 ml of cell-free lavage effluent (pooled from two rats) was centrifuged at 49,000 *g* for 30 min. The resulting sediment was resuspended in 3 ml of HBSS; this suspension is referred to as ALM.

H-ALM was obtained in one of two manners. Four patients underwent whole lung lavage (14) under general anesthesia for therapeutic reasons. After informed consent was obtained, seven patients had limited lavage through a fiberoptic bronchoscope with nonheparinized normal saline (24°C), and were injected with 50-ml aliquots through the bronchoscope with a syringe until there was suitable return with gentle aspiration. The volume of instilled saline varied between 150 and 500 ml, with recovery of 50–80%. Aliquots were taken for clinical diagnostic studies and H-ALM was then isolated and treated as described above.

ALM from both rats and humans was cultured for bacterial contaminants. Results of experiments utilizing contaminated R-ALM were discarded and repeated. However, results of experiments utilizing contaminated H-ALM were not discarded unless the contaminant was also present in the pour plates assaying intracellular viability.

Protein concentration of H-ALM was determined by the Lowry technique (15).

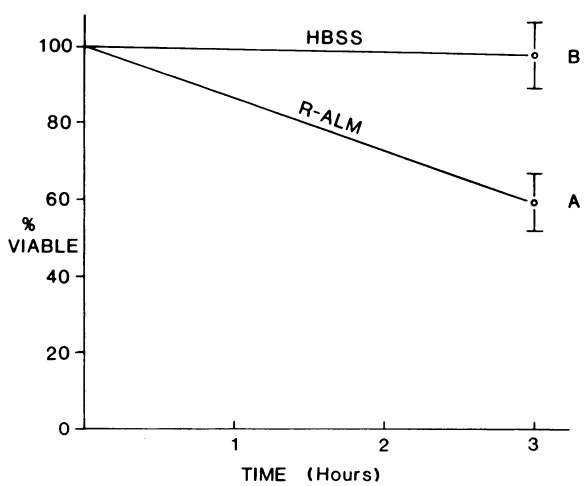

FIGURE 1 Intramacrophage viability of *S. aureus* which were incubated with HBSS or R-ALM before phagocytosis by rat alveolar macrophages is plotted. Base-line counts normalized to 100% viability. Line A is drawn to the mean 3-h count (expressed as percent of base-line count) of 10 experiments in which *S. aureus* was preincubated with R-ALM. Line B is drawn to the mean 3-h count of 10 matched experiments in which *S. aureus* was preincubated with HBSS (no ALM). Brackets mark ±SD.

Total lipids were determined on seven R-ALM samples and one sample of H-ALM by dry weight of chloroform: methanol extracts (16). The chloroform-soluble alveolar lipid fraction (ALF) was then resuspended in HBSS to yield a concentration of 0.1–2.5 mg/ml.

In vitro bactericidal assay. We used the technique of LaForce et al. (12), with some modifications. Basically, this quantitates the in vitro killing of phagocytized *S. aureus* by rat alveolar macrophages by measuring the number of viable intracellular bacteria as a function of time. In a series of 50 × 12-mm Petri dishes, monolayers of alveolar macrophages were suspended in HBSS, 10% fresh serum, and 10% strain-specific, heat-inactivated antisera (produced in rabbits, diluted 1 : 10) in a total volume of 0.9 ml. The alveolar macrophages (1×10^6) were incubated for 15 min at 37°C to allow attachment to the plastic dishes. *S. aureus* (50×10^6 cells in 0.1 ml) was added to each dish. Control monolayers received bacteria that have been preincubated in HBSS for 1 h at 37°C on a rotary shaker. Experimental monolayers received organisms pre-incubated in a suspension of R-ALM. The cell-bacteria mixtures were shaken at 20 cpm for 1 h in a 37°C incubator. After phagocytosis had proceeded for 1 h, extracellular *S. aureus* in all dishes were lysed with lysostaphin, a murolytic enzyme which is specific for staphylococci [2] and does not enter phagocytes (17). After 15 min, the enzyme was inactivated with 2.5% trypsin (0.1 ml) in the pairs of dishes which serve as base-line (or time 0) intracellular counts. After an additional 3 h of incubation, the viable intracellular bacteria in the remaining paired dishes were determined.

Counts of viable intracellular bacteria were determined in the following manner: cold distilled water is added to the Petri dishes, osmotically lysing the alveolar macrophages. An aliquot of the lysate is serially diluted in distilled water, and duplicate nutrient agar pour-plates prepared. All colony counts are performed after 48 h of incubation. Results are expressed as the percentage change in intracellular viability of staphylococci over the 3-h period.

When H-ALM was assayed and when ALF was assayed, controls consisted of rat alveolar macrophage monolayers and bacteria preincubated with R-ALM. If these control systems failed to demonstrate a change in viable intracellular bacteria within 2 SD of our previous experimental results, the experiment was discarded and repeated.

Including the assay of the ALF, a total of 38 experiments were performed. Results of four of these were discarded and repeated. Two were thrown out because of bacterial contamination of the R-ALM, and two were discarded because of technically poor quality with control values not within ±2 SD of our previous results. H-ALM from five patients was contaminated with flora from the upper respiratory tract; however, no contaminants were present in the assay pour plates.

Controls utilizing R-ALM, H-ALM, or ALF in a system containing 10% antisera, 10% rat serum, and HBSS showed no killing of *S. aureus* in the absence of alveolar macrophages.

Statistical methods. Calculation of arithmetic means and standard deviations were performed and comparisons made according to the Student's *t* test for unpaired data (18).

[2] Each lot of lysostaphin was assayed for lytic activity against our strain of *S. aureus* and consistently caused a 4–5 log drop in colony forming units, even when the bacterial inoculum was 20 times greater than that used in the monolayers.

TABLE I

Results of In Vitro Assay of Rat Alveolar Macrophage Bactericidal Capacity Utilizing ALM from Human Subjects

Patient	Disease	Lowry protein*	Change in viable intracellular bacteria
		μg/ml	%
1.‡ E. L.§	Squamous cell carcinoma, LUL	240	−65
2.‡ M. C.§	Nodule RUL (Old TB)	40	−54
3. A. R.§	Alveolar cell carcinoma	390	−43
A. R.‖	Alveolar cell carcinoma	100	
4. B. S.‖	PAP	860	−42
5. M. G.‖	PAP	670	−35.5
6. C. C.‖	PAP	560	−31.5
7.‡ H. M.§	Squamous cell carcinoma of mouth (RUL infiltrate)	N.D.	−30
8. D. S.§	Bronchiectasis	250	−5
9.‡ L. M.§	Oat cell carcinoma, RLL and RML	440	+0.5
10.‡ A. B.§	Bronchiectasis	N.D.	+2
11. L. B.§	Oat cell carcinoma, RLL	240	+15.5

LUL, left upper lobe; ND, not determined; PAP, pulmonary alveolar proteinosis; RLL, right lower lobe; RML, right middle lobe; RUL, right upper lobe.
* Concentration of ALM after resuspension in 3 ml HBSS.
‡ Bacterial contaminants present in ALM.
§ Segmental lavage.
‖ Whole lung lavage.

RESULTS

Bronchoalveolar lavage of rats yielded 2–5 × 10^6 cells per animal. Differential counts demonstrated 96±2.5% (SD) were mononuclear and morphologically consistent with alveolar macrophages. These cells were 94±2.4% (SD) viable on recovery, and no decline in viability was noted in the in vitro system for the duration of the experiment.

Our results comparing bactericidal capacity of rat alveolar macrophages with and without the addition of R-ALM are depicted in Fig. 1. This is a composite diagram representing the results of 10 experiments. The change in viable intracellular *S. aureus* incubated with R-ALM was − 39.1±9.4% (SD) and was significantly different from the intracellular viability of *S. aureus* incubated with HBSS, which declined only − 2.0±8.5% (SD) ($P < 0.001$).

H-ALM produced divergent results. Table I lists all the subjects from whom H-ALM was isolated, their primary clinical problem, the change in viable intracellular bacteria observed when H-ALM was substituted for R-ALM in the in vitro assay, and protein concentration of the H-ALM after resuspension in HBSS. The patients are numbered 1–11 according to the extent to which their H-ALM enhanced rat alveolar macrophage bactericidal capacity. H-ALM from patients 1–7 facilitated rat alveolar macrophage in vitro bactericidal function in a manner quantitatively similar to the homologous R-ALM. H-ALM from patients 8–11 failed to enhance in vitro rat alveolar macrophage bactericidal function.

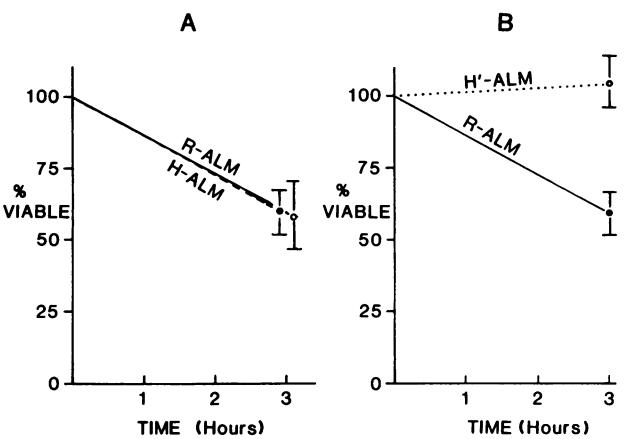

FIGURE 2 Graph A demonstrates the intracellular fate of *S. aureus* in rat alveolar macrophages. Base-line counts are normalized to 100% viability (ordinate) and the lines connect to the mean of the percent viable after 3 h. *S. aureus* was preincubated with H-ALM from subjects 1–7 in Table I with R-ALM as control. Brackets represent ±SD. Graph B again demonstrates the intracellular fate of *S. aureus*. *S. aureus* was preincubated with H'-ALM from humans 8–11 in Table I with R-ALM as control. Brackets represent ±SD.

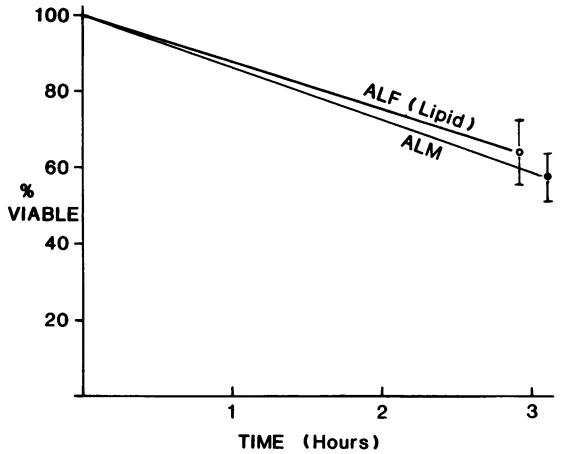

FIGURE 3 Intracellular fate of *S. aureus* in rat alveolar macrophages is shown. Base-line counts are normalized to 100% and mean 3-h counts (expressed as percent of base-line) of five experiments are shown. Line ALF indicates bacteria preincubated with alveolar lipid fraction, and line ALM indicates bacteria preincubated with crude ALM. Brackets represent ±SD.

Fig. 2A compares the in vitro bactericidal effect of H-ALM from patients 1–7, and Fig. 2B compares patients 8–11, plotted against control systems utilizing R-ALM. The change in viable intracellular bacteria was $-43\pm12.7\%$ (SD) with H-ALM from patients 1–7, compared to the R-ALM controls with a mean of $-40.5\pm7.6\%$ (SD). The change in viable intracellular bacteria seen with H-ALM from patients 8–11 was $+3.2\pm8.7\%$ (SD), which is significantly different from H-ALM from patients 1–7 ($P < 0.001$) and R-ALM ($P < 0.001$).

Preliminary results utilizing the ALF of ALM from one human subject and four rats indicate that the in vitro bactericidal effect can be demonstrated (viability $-37\pm8.4\%$ SD) and is virtually identical to that seen with the in vitro systems utilizing R-ALM. Fig. 3 compares the decline in viable intracellular bacteria observed with crude ALM and ALF.

DISCUSSION

The pioneering work of Laurenzi et al. (7), Green and Kass (8), and Green and Goldstein (19) suggested that alveolar macrophages are intimately involved in the intact lung's ability to clear inhaled microorganisms. Utilizing an aerosol generator, radiolabeled viable bacteria can be deposited in the alveoli of experimental animals. The decay in radioactivity then provides an index of clearance of bacteria, and serial quantitative bacterial cultures of lung homogenates provide an index of bacterial viability over time. The decline in bacterial viability in excess of the decline in radioactivity reflects bacterial killing by alveolar macrophages (8). Refinement of this concept has been provided by Johanson et al. (9), who demonstrated that in vivo bacterial replication must be considered, and that bacterial doubling times vary in response to lung injury. These observations, which suggested a major bactericidal role for the alveolar macrophage in vivo, contrasted with the observation that alveolar macrophages from guinea pigs and rats were unable to kill *S. aureus* in in vitro systems (10, 11). The dilemma seemed to be resolved when LaForce et al. (12) found R-ALM to be the independent variable, its presence being required in their in vitro assay. Without R-ALM, rat alveolar macrophages demonstrated no capacity to kill phagocytosed *S. aureus*. A similar phenomenon has been described in a system utilizing *Ps. aeruginosa* (13). The results of the present study support these observations; a decline in viable intracellular *S. aureus* is seen only with the inclusion of ALM in the system. Whether H-ALM could enhance the bactericidal activity of rat alveolar macrophages had not previously been studied. In the present study, H-ALM was isolated from the lung washings of 11 human subjects. These samples then were employed in the in vitro assay system. The effect of each subject's H-ALM on rat alveolar macrophage bactericidal capacity was compared with that seen with R-ALM. H-ALM from seven subjects produced enhancement of bacterial killing comparable to that observed with R-ALM controls. H-ALM from the remaining four subjects yielded no enhancement of bacterial killing by rat alveolar macrophages. The effectiveness of the H-ALM did not correlate with the chest X rays or smoking history. The incidence of two patients with bronchiectasis and two patients with oat cell carcinoma in the second group may merely be coincidence. The more exciting possibility is that altered macrophage bactericidal activity, whether due to altered alveolar lining material or due to other factors, might be etiologically related to bronchiectasis. Such speculation must be tempered by a recognition that inflammatory by-products may inhibit macrophage activity so that the failure to enhance macrophage bactericidal capacity in vitro may be a result, rather than a cause, of bronchiectasis. Likewise, the number of patients is too small to warrant conclusions and further investigation is needed.

Whether the factor(s) responsible for the demonstrated enhancement reside in the lipid, protein, or carbohydrate fractions of the ALM is not yet known. Although specific proteins were not characterized in the present study, no correlation was observed between protein concentration and effectiveness of H-ALM in the in vitro assay. Suggestive evidence that a protein may not be the major factor responsible for the observed enhancement is the preliminary observation that enhancement of rat alveolar macrophage bactericidal capacity can be

produced using the chloroform-soluble (lipid) extract of ALM from both rats and humans. No protein was detectable by Lowry methods in this ALF. Though limited to five experiments, the results with ALF have been reproducible and quantitatively comparable to those seen with crude ALM. Further investigation into the role of ALF, as well as further studies to delineate any nonlipid substances in ALF, are currently being pursued.

The mechanism by which ALM facilitates bacterial killing by alveolar macrophages is unknown. The results of this study demonstrate that the observation is reproducible with R-ALM, that heterologous H-ALM from some humans produces a similar effect, and suggest that the responsible factor is in the chloroform-soluble extract of the ALM. With a refined understanding of the mechanism responsible for these observations, testing the system with other bacterial pathogens, and the eventual inclusion of human alveolar macrophages, it may be feasible to develop an in vitro assay that would allow a more direct means of studying factors impairing human lung defense mechanisms against bacterial infection.

ACKNOWLEDGMENTS

This work was supported by grants from the National Institutes of Health, HL 01017-01 and AI-00326, and by funds from the Veterans Administration Hospital, Oklahoma City, Okla.

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