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THE ROLE OF ALVEOLAR MACROPHAGES IN THE PRODUCTION OF COLONY-STIMULATING FACTOR BY THE LUNG

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ABSTRACT

The role of alveolar macrophages in the production of granulocyte/macrophage colony-stimulating factor(s) by the rat lung was investigated. Lavaged lungs, when incubated at proper weight per volume of culture medium, produced the same amount of colony-stimulating factor as unlavaged ones. Both lavaged and unlavaged lungs produced similar types of colony-stimulating factor (s). Prolonged incubation of lavaged and unlavaged lung tissues did not result in higher levels of activity beyond that produced by 48 hrs of incubation. Alveolar macrophages recovered from the lung did not produce colony-stimulating factors when they were cultured unstimulated and under similar conditions as lung conditioned medium. The results indicated that alveolar macrophages did not play a significant role in colony stimulating factor (s) production by the lungs and when unstimulated, they did not produce colony-stimulating factors directly.

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INTRODUCTION

Colony-stimulating factors (CSF) control the *in vitro* growth and differentiation of hematopoietic progenitor cells.[1] There are four major identified subgroups of biochemically distinct CSF in the murine system. These are multi-CSF or interleukin-3, granulocyte/macrophage CSF (GM-CSF), macrophage CSF (M-CSF or CSF-1), and granulocyte CSF (G-CSF). These factors have been prepared from a variety of tissues and cellular sources [2] The medium conditioned by the lung has been st idied extensively and it contains mainly GM-CSF and some G-CSF.[3] The cell type(s) responsible for the production of CSF by the lung has not been determined.

Lung is a heterogenous tissue with different cell types consisting of alveolar macrophages (AM), endothelial cells, epithelial cells, and interstitial cells. Each of these cell types contribute differently to the CSF production by the lung.

Initially tissue macrophages and blood monocytes were believed to produce CSF.[4] Recent studies have indicated that while the stimulated tissue macrophages and monocytes produced CSF,[5] the unstimulated cells did not directly produce any colony-stimulating factor. They required the aid of accessory cells to exert their colony-stimulating activities.[6]

We have studied the role of AMs in CSF production by the lung *in vitro*, using broncho-alveolar lavage, a technique commonly employed to remove AMs from mammalian lungs.

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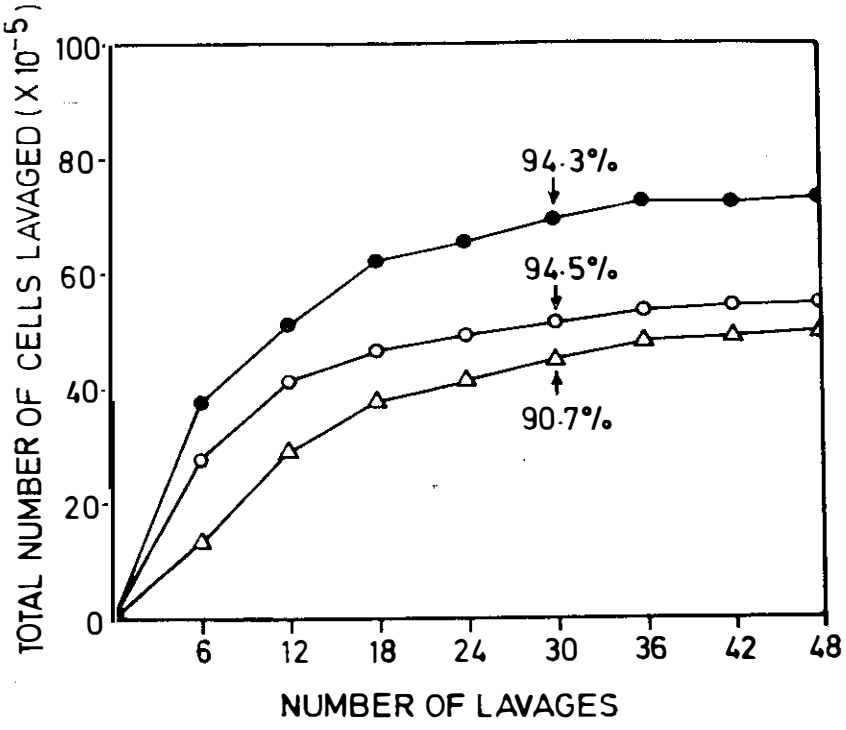

Fig 1. The washout pattern of AM cells. Three representative animals are shown. Arrows and their figures indicate fraction of cells obtained by 30 lavages.

MATERIALS AND METHODS

Animals: Sprague-Dawley rats, 250-350 g, were used in all experiments for the preparation of lung conditioned medium. Balb/c mice, 20-25 g, were used as the source of bone marrow cells.

Broncho-alveolar lavage: Rats were anesthesized by ether. The chest cavity was opened and the trachea was cannulated without any attempt to isolate the lung from the chest cavity. Each lavage consisted of 5 times infusion of 10 ml sterile normal saline supplemented with 1000 mg/liter of glucose at room temperature. Lungs were lavaged several times according to the experimental protocol. Lavaged lungs were treated for the preparation of conditioned medium as will be described later.

Lung conditioned medium: Lavaged or unlavaged lungs were removed from the chest cavity and were rinsed with normal saline. 0.5 g of unlavaged or 1.5 g of lavaged lung tissue was minced and incubated in 5ml of Dulbecco's MEM (GIBCO), supplemented with 30 mg/liter of Asparagine and antibiotics (200 U/ml penicillin and 200 mg/ml streptomycin), for 48 hrs at 37°C in fully humidified atmosphere containing 5% CO_2. In one group of experiments different weights of lavaged or unlavaged lung tissue were incubated in 5 ml of culture medium. In another group of experiments lung tissue was cultured for longer than 48hrs according to the experimental protocol. The conditioned medium thus obtained was collected, centrifuged for 30 minutes at 3000 g at 4°C and dialyzed against two changes of distilled water at 4°C for 48 hrs. The dialyzed conditioned medium was centrifuged as it was described and the clear supernatant was used as the source of CSF without further purification.

Alveolar macrophages: Cells recovered from the lavages were pooled and centrifuged at 1500 g for 15 minutes at 4°C. The pellet was washed once with normal saline, cells were counted, using a hemocytometer and their viability were determined by the trypan-blue exclusion test. Cytocentrifuge preparations, stained with Wright and Giemsa, were employed to identify macrophages.

Conditioned medium from AM was prepared by incubating various number of AMs in Dulbecco's MEM supplemented with 10% fetal calf serum (prepared in our laboratory) and antibiotics for 30 minutes or 24 hrs either in 80 mm glass petri-dishes containing a total of 10 ml, or in 55 mm plastic petri-dishes (NUNC) containing a total of 5 ml of culture medium. The non-adhering cells were thoroughly removed and the adhering layer consisting of 10-25% of original cells with more than 90% macrophages, were washed twice with normal saline. Finally, they were incubated in 55

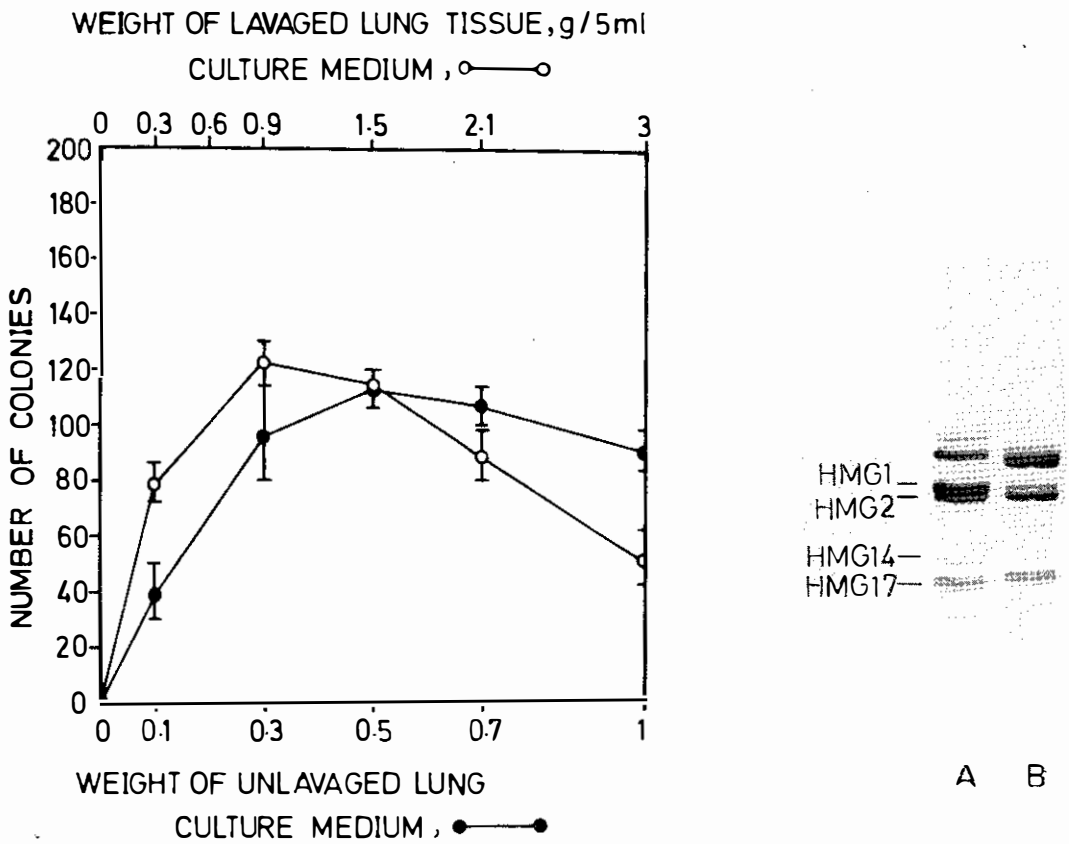

Fig 2. The effect of lung tissue weight cultured on CSF production. Various amounts of lavaged and unlavaged lung tissue were incubated in 5ml of the culture medium. Each data point represents mean ±SD of two experiments with duplicate plates.

mm plastic petri-dishes in Dulbecco's MEM (without fetal calf serum) for various periods of times according to the experimental protocol. The conditioned medium thus obtained was treated in the same manner as the lung conditioned medium.

CSF bioassay: Polyethylene glycol was added to the conditioned medium at a final concentration of 1% before it was sterilized by filtration through 45μm membrane filters (Millipore). The samples were assayed by semi-solid agar culture technique.[3] 10^5 bone marrow cells from Balb/c mice were plated into 35 mm plastic petri-dishes (NUNC) containing 1 ml of Dulbecco's MEM, supplemented with 0.3% agar, antibiotics, 30 mg/liter of Asparagine, and 20% fetal calf serum in the presence of 0.1 ml of the conditioned medium. Seven days after incubation at 37°C in fully humidified atmosphere of 5% CO_2 the colonies containing 50 or more cells were scored under a dissecting microscope.

To analyze the colony types, the colonies were fixed with absolute methanol for 20 minutes. The agar gel was cut in halves and they were transferred on microscope slides. After drying, the slides were stained with 0.6% orcein in 60% acetic acid for 1 hour.

Protein determination: The total protein contents of the samples were determined according to the procedure of Lowry modified by Hartree[7] using bovine serum albumin (Sigma) as standard.

RESULTS

The washed out pattern of AMs for 48hr lavages from three rats are illustrated in Figure 1. Progressively fewer cells were recovered by each lavage. The number of AMs in each lavage and the total number of cells recovered from each animal was different. In every case the first 30 lavages contained more than 90% of maximum number of cells recovered from the lung; therefore, in all subsequent studies, each lung was lavaged 30 times or less as indicated. Stained cytocentrifuge preparations of cells collected from the lung contained 80-85% AM, and remaining cells were neutrophils, lymphocytes, exfoliated epithelial cells, followed by some mast cells. Viability test usually indicated more than 94% viable cells.

The colony-stimulating activity in the lung conditioned medium depended on the weight of the lung tissues incubated per 5 ml of the culture medium. The

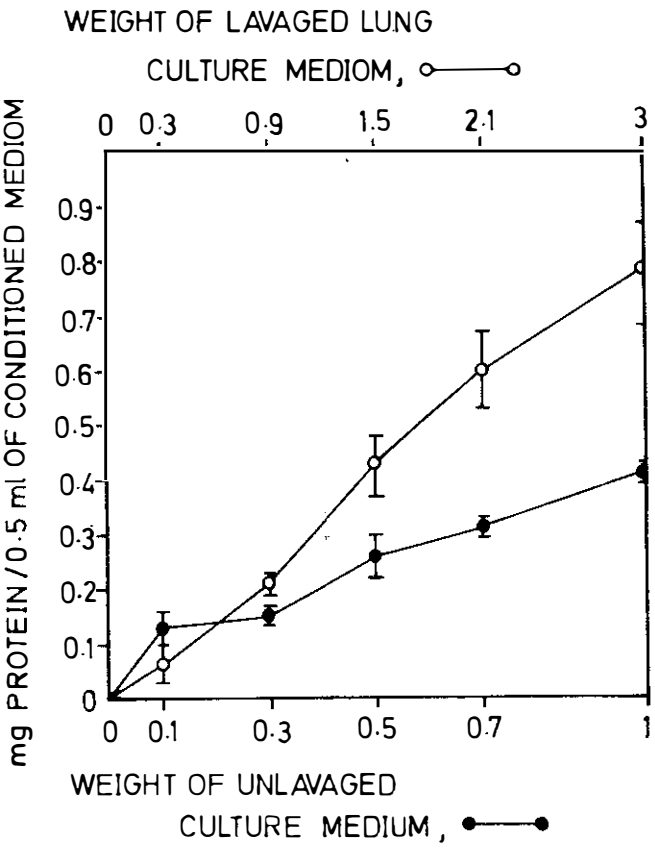

Fig 3. The total protein content of lavaged and unlavaged lung conditioned medium as a function of the lung tissue weight incubated in 5ml of the culture medium. Data represented corresponds to samples explained in Fig. 2. Same statistics as Fig. 2.

process of lavaging always resulted in the remaining of residual saline in the lung; therefore, the lavaged lung weighed heavier than the unlavaged ones. In order to correct and to determine the proper weight of lavaged lung incubated per 5 ml of the culture medium, it was necessary to incubate various amount of lung tissues per 5 ml of the culture medium. The results are shown in Figure 2 for both lavaged and unlavaged lungs. The maximum CSF activity of unlavaged samples were obtained at 0.5 g lung tissue per 5 ml of the culture medium. Incubation of smaller or larger amounts of tissues resulted in decreased activity. The peak of CSF activity for lavaged samples were observed at 0.9 g of tissue per 5 ml of the culture medium. The two activity curves from lavaged and unlavaged lungs intersected at 1.5 g of lavaged and 0.5 g of unlavaged tissues. Therefore, in all subsequent studies 0.9-1.5 g of lavaged tissue were incubated against 0.5 g of unlavaged tissue per 5 ml of the culture medium. The corresponding total protein contents from both lavaged and unlavaged samples are shown in Figure 3. Both curves increased linearly as the weight of lung tissues incubated were increased.

In order to determine the role of AMs in the CSF production by the rat lung, various numbers of the cells were removed from the lung and the CSF activities in the corresponding conditioned medium were determined. The results are shown in Figure 4. The number of AMs recovered from each rat with a certain number of lavages was different, hence the results are expressed in terms of the number of lavages performed. The fractions of AMs removed from each group of lavages were approximately the same for each animal. There was random variations in the number of colonies stimulated by the conditioned medium from lavaged lungs. No statistically significant ($p < 0.01$,

Table I. Fractions of granulocytic, G; macrophage, M; and mixed granulocytic macrophage, G-M; colonies from lavaged and unlavaged lung conditioned medium. Mean± SD.

Sample	%G	%M	%G-M
Unlavaged n=4	4.25±0.9	56.47±8.4	32.47±7.5
Lavaged n=3	6.46±1.95	65.5±4.3	27.8±2.7
d[1]	1.608	1.851	1.03

1- Two sample t-test with unequal population variance, $P<0.01$.

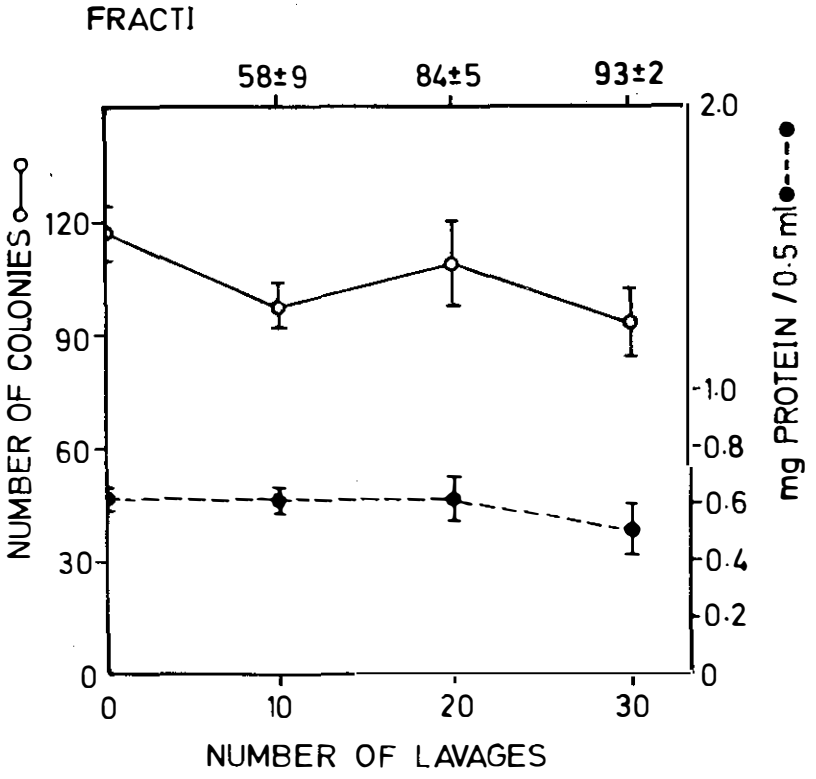

Fig 4. The CSF and the total protein content of the conditioned medium from the lungs lavaged various number of times. The number of cells recovered by lavaging differed for each animal; therefore, data is represented in terms of the number of lavages rather than the number of cells recovered. The fraction of cells removed from the lung by each number of lavages are fairly similar for various animals and it is indicated on the top of the figure. Mean±SD of five experiments with duplicate plates.

Student t test) difference was observed between the total protein contents of lavaged and unlavaged conditioned medium.

The colony typing was performed to examine the nature of CSF produced by the lavaged and unlavaged lungs. The results are given in Table I. The distribution of colonies from both samples were similar, indicating the similarities between the types and the amount of CSFs produced by lavaged and unlavaged lung tissues.

The 48 hrs incubation experiments showed no significant difference in the CSF level of lavaged or unlavaged lung conditioned medium; therefore, a time course study was performed to examine the effect of prolonged incubation of CSF production. For this purpose lavaged and unlavaged lung tissues were incubated from two to seven days and samples were taken at various intervals. The results are shown in Figure 5 for both samples. The total protein contents of samples are also shown in Figure 5. No significant differences were observed between the activities of lavaged and unlavaged samples in either 48 hrs or 7 days incubations. The total protein contents from both samples remained fairly constant during the prolonged incubation. The lavaged samples contained consistently, but insignificantly ($p<0.01$) less protein than unlavaged ones.

To evaluate directly the ability of alveolar macrophages to synthesize CSF *in vitro,* various numbers of these cells ranging from 1×10^6 per 5 ml to 20×10^6 per 10 ml of culture medium were incubated from 24 hrs to 20 days as described in the method section. None of the AM conditioned media prepared contained any detectable colony stimulating factors.

DISCUSSION

The inability of unstimulated macrophages to produce CSF has been reported by other investigators. The reports which have claimed CSF-production by unstimulated macrophages have used either macrophages which were derived from *in vitro* differentiation of blood monocytes[8] or from the crude preparations of tissue macrophages.[4,9] Although macrophages which were derived from *in vitro* differentiation of monocytes resembled the tissue macrophages, they apparently did not thoroughly represent unstimulated tissue macrophages. It has been shown that pure unstimulated preparations of blood monocytes were not able to produce detectable amounts of CSF in the conditioned medium.[10]

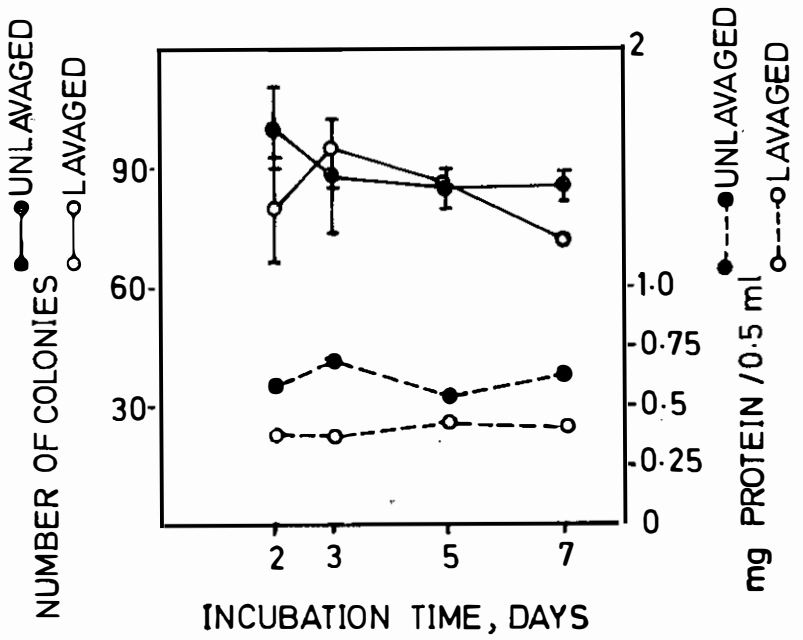

Fig 5. Time-course study of CSF production by the lung. Lavaged or unlavaged lung tissues were incubated from 24 hrs up to 7 days. Mean±SD of two experiments with duplicate plates.

The crude preparations of tissue macrophages contained 50-90% macrophages, depending on the tissue source. The rest of the cells are mostly lymphocytes and endothelial cells which are known to be able to produce CSF. Purified macrophages from these populations were not able to produce CSF.[5]

In conclusion, the results presented here, in agreement with the prior results, indicate that AMs neither produce CSF directly, nor play a significant role in the CSF prodution by the lung. Therefore, the question of which cell(s) are responsible for the CSF production by the lung remains to be answered.

ACKNOWLEDGEMENTS

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