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FEEDBACK REGULATION OF COLONY-STIMULATING FACTOR PRODUCTION

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ABSTRACT

The production of colony-stimulating factors (CSF) is delicately controlled through a complex network of humoral and environmental factors. We have studied some of the mechanisms which regulate the production of CSF as compared to general protein synthesis in the lung tissue *in vitro*. When lung tissue from mice was cultured for various times in serum free medium, the first detectable level of CSF activity in the lung conditioned medium (LCM) appeared 6 hr after initiation of the culture, continued to rise until 24 hr, and then levelled off for several days. Under similar conditions protein synthesis did not level off, but continuously rose after 24 hr. When the lung tissue which had been cultured previously for 6, 24, or 48 hr was recultured in fresh tissue culture medium, *de novo* synthesis of CSF occurred as judged by CSF synthesis inhibition and stimulation studies. The amount of new CSF synthesized by these tissues decreased as the initial culturing period increased from 6 to 48 hr. There was also a decrease in the amount of total protein synthesis and release in the secondary lung cultures as a function of the initial culturing period. Endotoxin stimulation of 24 or 48 hr-cultured lung tissues (plateau phase tissue) resulted in *de novo* synthesis of CSF by these tissues. However, when fresh lung tissue was cultured in 24 or 48 hr LCM, no new CSF was produced by the fresh tissues, while under similar conditions, protein synthesis by these tissues was significant as judged by double-labelling experiments. On the other hand, 6 hr LCM was able to support both CSF production and protein synthesis by fresh lung tissues. The results suggest at least two distinct regulatory systems controlling CSF production by the lung *in vitro*: 1- Aging which is responsible for general and nonspecific decrease in the rate of protein synthesis and CSF production in this system, and 2-Feedback regulation of CSF production by the level of CSF which is formed in the LCM.

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INTRODUCTION

Colony stimulating factors (CSF) are humoral regulators of hematopoiesis. Historically, they were named after the specific cell lineage which they could support and colony formation in the semi-solid cultures of bone marrow.[1,2] The four known CSF; G-CSF, GM-CSF, M-CSF, multi-CSF, and the recently assigned Il-6 collectively control various stages of blood cell formation from early differentiation of stem cells to functional ability of mature blood cells.[3] Within the last decade a large body of information has been generated about the molecular biology, production, and the application of recombinant CSF.[4-11] However, many fundamental questions about the physiological regulation of natural CSF production has gone unattended.

Blood serum of animals and man contains basal levels of CSF.[12-15] The tissue source(s) of this serum CSF is not known.[16] However, many cell types and tissues have been shown to produce CSF *in vitro*.[17,18] The lung is of special interest because it is the most potent tissue source of CSF *in vitro*.[19] Some authors have shown variations in CSF levels in various pathological conditions.[14,15,20-22] Various physical and chemical factors also affect the production of CSF by the lung and other tissues or cell lines *in vitro*.[22-27] These data indicate that the production of CSF should be controlled through a multi-component and complicated system. However, no data exists that might indicate a feedback regulation of CSF production by the CSF molecules.

In this work we have studied the possible feedback regulation of CSF production by the tissue as a model system.

MATERIALS AND METHODS

Animals: Sprauge-Dawly rats of either sex weighing 200-300 g were used for the preparation of LCM and CSF. Bone marrow cells, used for CSF bioassay, were obtained from Balb/c mice weighing 18-20 g.

Lung conditioned medium: The LCM was prepared by incubating 0.5 g of finely minced lung tissue in 5 mL of Dulbecco's modified Eagle medium (DMEM) supplemented with 30 mg/L of asparagine and antibiotics (200 U/mL penicillin and 200 mg/mL streptomycin) in 50 mm plastic petri dishes (NUNC) in a fully humidified atmosphere of 5% CO_2/95% air at 37°C for various times as indicated in the experimental protocol.

CSF preparation and bioassay: The LCM was collected, centrifuged for 30 min. at 3000 g at 4°C, and heated at 56°C for 30 min. It was then centrifuged as above and the supernatant was dialyzed against two changes of distilled water at 4°C for 48 hr. The dialyzed LCM was centrifuged at 10,000 g and 4°C for 1 hr and the clear supernatant was used as the source of the CSF without further purification. Polyethylene glycol at the final concentration of 1% was added to the LCM before it was sterilized by filtration through 0.45 µm membrane filters (Millipore). The biological activity of samples were assayed by the semi-solid agar culture technique.[2] 10^5 bone marrow cells were plated into 35 mm plastic petri dishes (NUNC) containing 1 mL of DMEM supplemented with 0.3% agar, antibiotics, 30 mg/L asparagine, and 20% fetal calf serum in the presence of 0.1 mL of the LCM. Seven days after incubation at 37°C in a fully humidified atmosphere of 5% CO_2, the colonies containing 50 or more cells were scored under a dissecting microscope.

Reculturing experiments: Two types of reculturing experiments were performed to set up secondary cultures. In one group of experiments the lung tissue was cultured for a given time, after which the tissue was removed, rinsed properly, and then cultured in fresh DMEM culture medium for 48 hr. In another group of experiments the lung was cultured for a given time, after which the LCM was collected. This LCM was supplemented with a labelled amino acid. Then a fresh piece of lung tissue was cultured in this LCM.

Protein biosynthesis: Protein biosynthesis was determined by the amount of incorporation of ^{14}C-labelled L-Leucine. 50 µCi/mL and final activity of 0.238 µCi/mL (Amersham), in the high molecular weight and nondialyzable fraction of the LCM. In double labelling experiments ^{3}H-labelled L-Leucine, 5 MCi/mL and final activity of 20 µCi/mL (Amersham) was used. Radioactivity incorporated into the LCM was measured by liquid scintillation counter. Results were expressed as cpm/mL of the LCM.

Protein determination: The total protein content of the LCM samples was determined according to the procedure of Lowry modified by Hartree.[28]

RESULTS

CSF production and protein synthesis by primary cultures of lung

The lung tissue was cultured as described in the METHODS section and at 6, 24, or 48 hr, samples were taken from the LCM and the CSF activity, total protein content, and the total protein synthesis for each sample was determined. The result is shown in Fig. 1. The first detectable level of CSF activity appeared 6 hr after initiation of the culture, continued to rise until 24 hr and then levelled off. In contrast, protein synthesis continuously rose over this period and did not show any plateau. The total protein content of samples did not vary significantly.

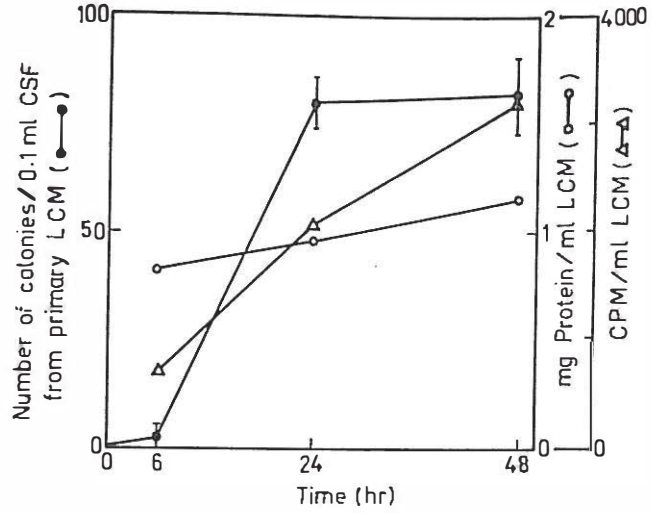

Fig. 1. The effect of incubation time on the production of CSF (●) and protein synthesis (△) by lung tissue and the total protein content of the LCM (○).

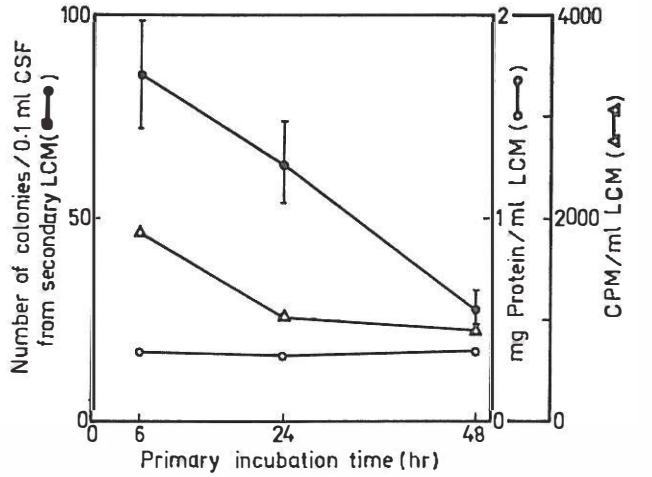

Fig. 2. The effect of primary incubation time on the production of CSF and protein synthesis by the lung tissue in the secondary culture. The number of colonies produced by the lung tissue in the secondary culture (●) is plotted versus the primary incubation time of the lung (△), and the total protein content of the secondary LCM (○) is shown as a function of the primary incubation time of the lung tissue.

CSF production and protein synthesis in secondary cultures

In this section we studied the ability of precultured lung tissues to produce CSF and synthesize protein upon transfer to fresh culture medium. Lung tissues were cultured in DMEM for various times as described in the previous section. Then at 6, 24, and 48 hr post-primary incubation, the tissues were removed, rinsed with saline, transferred to fresh DMEM, and incubated for 48 hr. At the end of the secondary incubation time the CSF produced, total proteins synthesized, and the total protein content of secondary LCM were determined. The results are shown in Fig. 2. Here the CSF activity, total protein content, and total protein synthesized is plotted versus the first incubation period for each sample. It can be seen that all three lung samples which were initially incubated for various times were able to synthesize CSF again in the secondary cultures. However, there was a reduction in CSF production in these cultures as a function of the primary incubation times of the lung tissues. Under similar conditions, protein synthesis was active. Here again there was a reduction in protein synthesis in secondary cultures as a function of incubation times of lung tissues in primary culture. However, the decline in protein synthesis from 24 hr samples to 48 hr ones was much less than that of CSF production. The total protein content did not show any difference in various samples

Effects of LPS: This experiment was designed to examine the ability of lung tissues in the plateau phase of CSF production to further synthesize CSF. For this purpose the tissues were cultured in DMEM and were allowed to reach the plateau phase by incubating them for 24 or 48 hr. The tissues were then removed, rinsed with saline, and cultured in fresh DMEM containing 0.2 μg/mL LPS for 48 hr. Controls did not receive any LPS. The results are given in Fig. 3. It can be seen that lung tissues which were in the plateau phase of CSF production and were transferred to fresh culture medium were able to start synthesizing CSF again. The extent of this novel CSF production can be enhanced by stimulation with LPS. These results and those of Fig. 2 suggested the idea that the plateau phase of primary cultures is not due to exhaustion of the ability of lung tissues to make CSF. Rather, there should be some sort of regulatory mechanism controlling the production of these CSF.

Effects of inhibitors of protein synthesis: Since it was possible that the novel CSF produced in the secondary cultures of lung tissues might be due to the release of preformed CSF from these tissues, the following experiment was performed to examine this possibility. In this regard, the lung tissue was cultured in DMEM for various incubation times of 6, 24, or 48 hr. At the end of this time the tissues were removed, rinsed with saline, and transferred to fresh DMEM containing puromycin, an inhibitor of protein synthesis, and cultured for 48 hr. Controls received no puromycin. The results are shown in Fig. 4. It can be seen that under these conditions no novel CSF synthesis has occurred in the secondary cultures.

Effects of preformed CSF on the novel production of CSF by the lung: We studied the ability of LCM with various CSF activities to support the *de novo* synthesis of CSF by fresh lung tissue. For this purpose LCM was

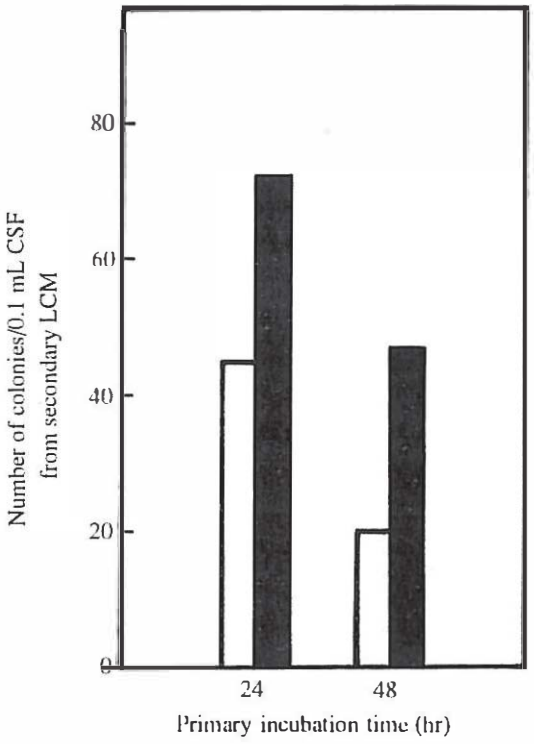

Fig. 3. The effect of LPS on the production of CSF (■) by the lung in the secondary culture as a function of the primary incubation time of the lung tissue (□= controls).

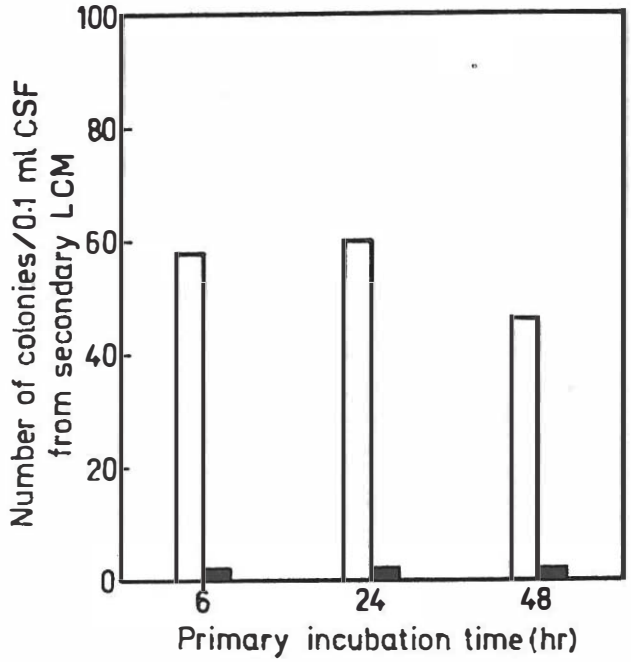

Fig. 4. The effect of puromycin, an inhibitor of protein synthesis, on the production of CSF (■) by the lung tissue in the secondary cultures as a function of the primary incubation time of the lung tissue. (□= controls).

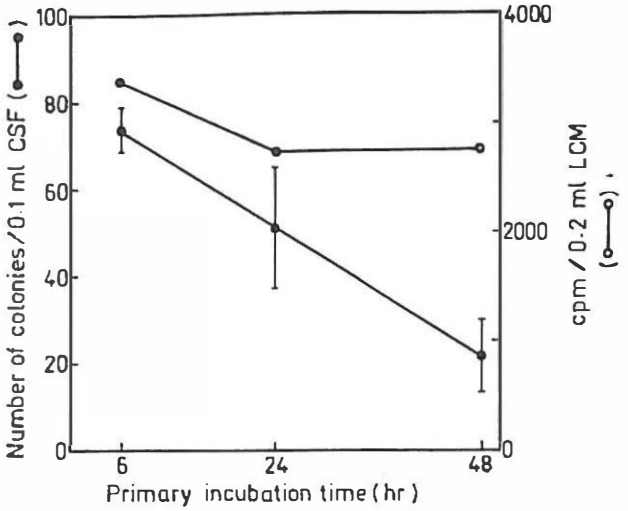

Fig. 5. The effect of preformed CSF on the production of CSF and protein synthesis by the lung tissue. Different samples of LCM were prepared by incubating the lung tissue in serum free DMEM for various times indicated on the abscissa. Then the lung tissues were discarded and the LCM was supplemented with ^{14}C-leucine. Fresh lung tissue was cultured in these LCM for 48 hr after which the amount of CSF (●) and protein synthesis (○) was determined.

prepared by incubating lung tissue for 6, 24, and 48 hr in serum-free DMEM supplemented with ^{3}H-labelled leucine. At the end of incubation time the lung tissues were discarded and the protein synthesis and CSF activity were determined in each LCM sample as described. Then ^{14}C-labelled leucine was added to each LCM and subsequently, 0.5 g of fresh lung tissue was incubated in 5 mL of each of these LCM samples for 48 hr. Finally, CSF activity and protein synthesis in secondary cultures were determined by the number of colonies produced and the amount of ^{14}C-labelled leucine incorporation. The results are shown in Fig. 5. In this figure, the number of colonies formed in the secondary LCM and the amount of radioactivity incorporated in these samples is plotted versus the primary incubation time of these LCM samples. It is evident that no new CSF was produced during incubation of fresh lung tissues in preformed LCM. Actually there was a decrease in CSF activity as the primary incubation time of LCM increased. Meanwhile, the protein synthesis by fresh lung tissue in preformed LCM, as judged by ^{14}C-leucine incorporation, was maintained constant with slight variations in some samples.

DISCUSSION

We have studied some of the regulatory mechanisms which regulate and control the production of CSF. The

hemopoietic system of animals is under the control of a delicate and highly complicated system. This system includes environmental and humoral components. A large body of information has become available on how these components impart their regulatory effect on the hemopoietic system. The humoral regulators which include CSF and other cytokines have been well characterized. Although some very fundamental questions on how the production of regulators themselves are controlled in the body have gone unattended, some *in vivo* observations indicate that the CSF level of serum is increased under pathologic conditions.[14,15] There is also evidence that some stimulator can alter the amount of CSF production *in vitro*.[22-24] However, so far no data has been available as to whether the CSF level in the medium can regulate its own production or not. We have addressed this question in this paper by using the lung culture as a model system for production of CSF. The results presented here indicate that CSF production is possibly controlled via a feedback mechanism.

When the lung tissue is cultured there is initially a lag of about 6 hrs before any CSF can be detected in the medium. Afterwards the CSF level rises in the medium up to 24 hrs and then remains fairly constant for several days. Exactly what causes the initial stimulation of CSF production is not known. However, it is clear that the CSF production is halted after 24 hrs in the culture (Fig. 1).

Various treatments revert the paused mode of CSF production. Transfer of lung tissue to fresh culture medium causes them to restart CSF production. Addition of stimulation such as LPS enhances this phenomenon (Fig. 3). These results indicate that after 24 hrs of lung culture the ability of lung tissues to produce CSF is not really exhausted. Therefore, other factors should be responsible for the pause in CSF production by the lung tissue. One such factor is the level of CSF itself which is formed in the conditioned medium. When lung tissues which already had been cultured for 6, 24, or 48 hr were transferred to fresh conditioned medium, they were able to start synthesizing CSF again (Fig. 2). This *de novo* synthesis of CSF could be totally inhibited by puromycin which is an inhibitor of protein synthesis.

Independent additional support for this proposal comes from the experiments in which lung tissue was cultured in LCM. In this case the *de novo* synthesis was totally inhibited in 24 or 48 hr LCM (Fig. 5). CSF constitute a very small fraction of proteins which are synthesized by the lung tissue and since the synthesis of other proteins by the lung is not altered in these experiments, it can be concluded that their synthesis is not regulated in the same manner as the synthesis of CSF.

In conclusion the data presented here suggest that CSF production by the lung is regulated by several factors including various stimulators and inhibitors. Among these the CSF itself imparts a negative feedback control on its own production. The exact molecular details of this new regulatory mechanism are not known at this time.

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