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Rapid Effect of Lymphokines on Macrophage Activities Determined by Measuring Chemiluminescence

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The effect of lymphokines on macrophage activities was studied by measuring the phorbol myristate acetate-induced luminol-dependent chemiluminescence. When thioglycolate-elicited BALB/c mouse peritoneal macrophages were incubated with lymphokines for a short time (4 h), their ability to generate chemiluminescence significantly increased, while more prolonged treatment (24 h) was less effective. These results show that the measurement of macrophage chemiluminescence is useful for rapid determination of lymphokines.

Keywords—lymphokine; macrophage; chemiluminescence; BALB/c mouse; luminol; phorbol myristate acetate; macrophage activating factor

Macrophage activating factor (MAF), a lymphokine which renders macrophages cytotoxic for tumor cells *in vitro*, is thought to play an important role in tumor immunity. However, the exact physicochemical characteristics and the true biological role *in vivo* of MAF have not yet been clarified, because the conventional method used for determination of MAF is semiquantitative, complicated and time-consuming, and also because MAF is only available in extremely small quantities. Thus, it is necessary to establish a quantitative, simple and rapid method for the determination of MAF for basic research and clinical application.

It is well known that macrophages activated by various stimuli release highly reactive oxygen metabolites, such as superoxide anion¹⁾ and hydrogen peroxide,²⁾ which are thought to be major effective molecules that mediate the killing of microorganisms³⁾ and tumor cells.^{4,5)} These active oxygens can be readily determined by measuring the luminol-dependent chemiluminescence.⁶⁾

Recently, Fischer *et al.*⁷⁾ and Ernst *et al.*⁸⁾ demonstrated that the ability of mouse bone marrow-derived macrophages to generate chemiluminescence increased after overnight treatment with macrophage cytotoxicity factor (synonymous with MAF in this paper). This chemiluminescence method seems to be most promising as a simple and rapid method for the determination of MAF.

In this report, we show that the ability of macrophages to generate chemiluminescence significantly increased after incubation with lymphokines for only 4 h, and that more prolonged treatment (24 h) was less effective.

Materials and Methods

Animals—BALB/c mice were purchased from the Shizuoka Laboratory Animal Center, Hamamatsu, Japan, and bred in our laboratory.

Reagents—Luminol (Wako Pure Chemical Ltd., Japan) was dissolved in 0.1 M carbonate buffer (pH 10.5) at a concentration of 10 mg/ml, and the solution was diluted to 2 mg/ml with 0.1 M phosphate buffer (pH 7.4) and stored at 4°C until use. Phorbol-12-myristate-13-acetate (PMA; Sigma Chemical Co., St. Louis, Mo.) was dissolved in dimethyl sulfoxide at a concentration of 1 mg/ml, and the solution was diluted to 200 µg/ml with distilled water and

stored in aliquots at -20°C until use. Both stock solutions were diluted to the desired concentrations with Hanks' balanced salt solution without phenol red (HBSS) immediately before use.

Instruments—In this investigation, most of the measurements of chemiluminescence were performed with inexpensive instruments at hand, a Chem-Glow photometer⁹⁾ (Aminco, Silver Spring, Md.) and an integrator-timer (NITI-ON Medical and Physical Instruments Mfg. Co., Ltd., Japan). In some experiments, a Luminescence Reader BLR-102 (Aloka Co., Japan) was used for measuring chemiluminescence.

Media and Culture Conditions—Unless otherwise indicated, cells were cultured in RPMI 1640 medium (Nissui Seiyaku Co., Japan) supplemented with 100 units/ml of penicillin, 100 $\mu\text{g}/\text{ml}$ of streptomycin and 10% heat-inactivated newborn calf serum (GIBCO, Grand Island, N.Y.). All cultures were performed at 37°C in a humidified atmosphere containing 5% CO_2 .

Preparation of Lymphokine Supernatants—Spleen cells of normal BALB/c mice were suspended in medium at a cell density of 5×10^6 cells/ml, and cultured with 5 $\mu\text{g}/\text{ml}$ of concanavalin A (Con A; Sigma) for 48 h. The culture was centrifuged, and the supernatant was retained as the source of lymphokines. As a control, a supernatant obtained from spleen cells cultured without Con A and then supplemented with Con A was used. Both culture supernatants were filtered through a 0.45 μm Millipore filter and stored at -80°C until use.

Preparation of Macrophages and Lymphokine Treatment—Macrophages were obtained from the peritoneal cavities of BALB/c mice that had been injected intraperitoneally with 2 ml of thioglycolate medium (TGC medium, fluid; Nissui) 4 d previously. These peritoneal exudate cells were suspended in medium and plated on plastic dishes (LUX, 60×15 mm) at 4×10^6 cells/dish. After a 1 h incubation, nonadherent cells were removed by washing twice with HBSS, and then the dish-adherent cells, regarded as macrophages, were incubated with 4 ml of media containing 10% lymphokine or control supernatants for 4 to 24 h.

Measurement of Chemiluminescence—Dish-adherent macrophages were washed twice with HBSS and collected by scraping with a Teflon blood spreader. These cells (about 2×10^6 cells) were centrifuged in a 5×50 mm glass tube and the pellet was suspended in 100 μl of HBSS. The tube was stood in a water bath at 37°C , and 100 μl each of prewarmed luminol (300 $\mu\text{g}/\text{ml}$) and PMA (3 $\mu\text{g}/\text{ml}$) solutions were added. The mixture was mixed well and placed in the reaction chamber of a Chem-Glow photometer at room temperature, and the chemiluminescence was counted for 30 s or 1 min with a NITI-ON integrator-timer. The operation of mixing and counting was repeated for 10 min or 20 min at approximately 1 min intervals. During the intervals between the chemiluminescence measurements, the reaction mixture was kept at 37°C in a water bath, because our instrument has no temperature control equipment.

In the case of the Luminescence Reader BLR-102, the sample volume was scaled up to 1.5 ml in polyethylene vials and the measurement of chemiluminescence was performed at 37°C with stirring. The results are expressed as the maximum values of integrated counts of chemiluminescence in 1 min.

Statistical Analysis—The statistical significance of differences was determined by Student's *t*-test. A *p* value of less than 0.05 was taken as being significant.

Results and Discussion

In preliminary experiments, we often failed to increase the ability of macrophages to generate chemiluminescence by overnight treatment with lymphokine supernatants. Concerning the induction of tumoricidal macrophages, Ruco and Meltzer¹⁰⁾ have already reported that mouse resident peritoneal macrophages treated with lymphokines for 4 h showed cytotoxic activity during a further 60 h incubation with tumor cells, but 24 h treatment was less effective. Thus, we measured the chemiluminescence generated from macrophages after not only 24 h, but also 4 h treatment with lymphokine supernatants.

Figure 1 shows the time course of PMA-induced chemiluminescence of macrophages after incubation with control or lymphokine supernatants. There is no difference between control and lymphokine-treated macrophages with respect to the pattern of chemiluminescence. However, the amount of chemiluminescence generated by macrophages which were treated with lymphokines for 4 h increased markedly in comparison with the control. On the other hand, 24 h treatment caused only a slight increase. A similar tendency, that a short (4–5 h) treatment with lymphokines was more effective for increasing the ability of macrophages to generate chemiluminescence than more prolonged (22–28 h) treatment, was observed in further experiments. In some experiments, we could not observe the effect of lymphokines on macrophage chemiluminescence at 24 h, although at 4 h the maximum chemiluminescence of lymphokine-treated macrophages was significantly increased ($n=4$, $p<0.001$) as compared

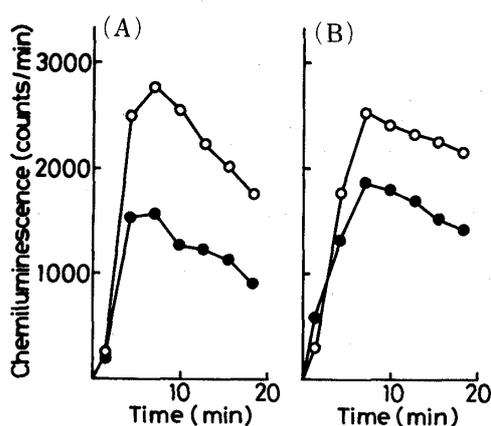


Fig. 1. Effect of Lymphokines on Macrophage Chemiluminescence

The time course of PMA-induced chemiluminescence of macrophages was examined after 4 and 24 h incubation with lymphokine (○) or control (●) supernatants. The chemiluminescent response to PMA of macrophages incubated with medium was similar to that of the control.

(A) 4 h. (B) 24 h.

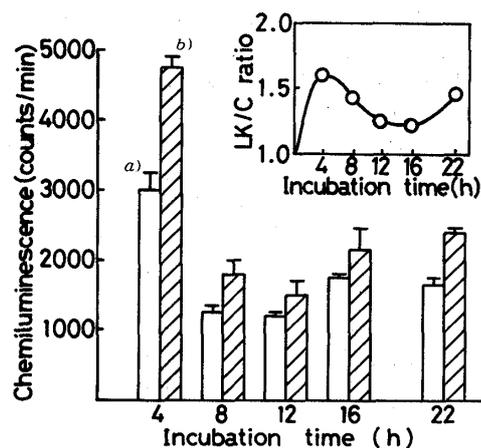


Fig. 2. Effect of Incubation Time with Lymphokines on Macrophage Chemiluminescence

Maximum chemiluminescence of macrophages was measured after various incubation times with lymphokine or control supernatants. Each bar represents the mean \pm S.D. of 3 determinations. The ratio of maximum chemiluminescence of lymphokine-treated macrophages to that of the control is shown in the inset. In this experiment, an Aloka Luminescence Reader BLR-102 was used.

a) Control. b) Lymphokine.

with the control.

As shown in Fig. 2, the effect of lymphokines on macrophage chemiluminescence was greatest at 4 h, and was reduced gradually with increase in the incubation time. In addition, an increase in the chemiluminescence of lymphokine-treated macrophages could be observed at only 2 h incubation (data not shown).

As to the time course of macrophage activation by lymphokines, a similar tendency was seen in the results obtained in the present experiments and those of Ruco and Meltzer. However, unlike their cytotoxicity test, our chemiluminescence method can determine the effect of lymphokines on macrophage activities immediately after lymphokine treatment without further incubation.

At present, as indicators of macrophage activation by lymphokines, bactericidal and tumoricidal activities or glucose consumption,¹¹⁾ *etc.*, are used, but at least 1 or 2 d are necessary to complete the assay. In this report, we show that the effect of lymphokines on macrophage activities can be determined after only a 4 h treatment by chemiluminescence measurement. These findings should contribute to the development of a rapid method for the determination of MAF, and furthermore to an understanding of the macrophage activation mechanism by MAF.

It should be noted that the luminol-dependent chemiluminescence system used in this report can not characterize the molecular species of active oxygens. Recently, Chiba and Kakinuma¹²⁾ developed an apparatus for the measurement of low level chemiluminescence, and tried to characterize the molecular species generated from polymorphonuclear cells by analyzing the emission spectrum using a luminol-independent chemiluminescence system. Such an approach should be suitable for the determination of the molecular species of active oxygens generated from activated macrophages.

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