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Biphasic Increase in Chemiluminescence of Lymphokine-Treated Macrophages

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The effect of incubation time with lymphokines on macrophage activities was studied by use of the phorbol myristate acetate-induced luminol-dependent chemiluminescence method and cytotoxicity test. When thioglycollate-elicited ICR mouse peritoneal macrophages were incubated with lymphokines, their ability to generate chemiluminescence increased biphasically during incubation. That is, within one day, it reached a maximal level at about 4 h (early response), and then progressively decreased to the control level. However, when the incubation time was further prolonged, it began to increase again, and reached about 2-fold the control level after 3 d (late response). The increase in the chemiluminescence of lymphokine-treated macrophages with increasing incubation time is not due to the increase in the macrophage cell numbers. In contrast to the clear biphasic increase in chemiluminescence, there was no clear biphasic increase in cytotoxicity in lymphokine-treated macrophages. The activities in the lymphokine supernatants to induce the early and the late chemiluminescent response in macrophages disappeared together with the activity to induce cytotoxicity, on dialysis at pH 2 for 24 h or on heating at 80 °C for 30 min, but not at 56 °C for 30 min. Although the lymphokines increasing macrophage chemiluminescence were separated into two fractions in Sephadex G-100 gel filtration, each fraction had both activities to induce the early and the late chemiluminescent response, and the major fraction largely corresponded to that of the activity to induce cytotoxicity. These results suggested that there is a lymphokine which alters macrophage chemiluminescence biphasically by itself, and it may be interferon-γ.

Keywords——lymphokine; macrophage; chemiluminescence; biphasic response; mouse; luminol; phorbol myristate acetate; cytotoxicity; macrophage activating factor; interferon-γ

Introduction

It is well known that macrophages activated by various stimuli release highly reactive oxygen metabolites, such as superoxide anion[1] and hydrogen peroxide,[2] which are thought to be major effector molecules mediating the killing of microorganisms[3] and tumor cells.[4] These reactive oxygen species can be readily determined by measuring the luminol-dependent chemiluminescence.[5]

Recently, the chemiluminescence method has begun to be used for monitoring lymphokine-mediated macrophage activation, since Fischer *et al.* and Ernst *et al.* showed that the ability of mouse bone marrow-derived macrophages to generate chemiluminescence increased after overnight treatment with lymphokines.[6] In many studies,[7] macrophage chemiluminescence was measured after overnight incubation with lymphokines. However, the effect of the incubation time on the macrophage chemiluminescence has not been examined in detail so far.

Previously, we showed that the effect of lymphokines on macrophage chemiluminescence became maximum at about 4 h (early response), and then disappeared on further incubation,[8,9] but in those studies, the incubation time was limited within one day.

In a subsequent investigation, however, we observed that the increasing effect of lymphokines on macrophage chemiluminescence, having disappeared once, began to appear again when the incubation time was further prolonged (late response).

In this paper, we show that the ability to generate chemiluminescence of macrophages increased biphasically during incubation with lymphokines, but that the lymphokines could not be separated into two parts corresponding to the early and the late chemiluminescent response. We consider that there may be a lymphokine which increases macrophage chemiluminescence biphasically by itself, and it may be interferon-γ.

Materials and Methods

Animals——Male ICR mice, purchased from the Shizuoka Laboratory Animal Center, Hamamatsu, Japan, were used in most experiments at 5—10 weeks of age. In an experiment to prepare serum-free lymphokine supernatants for gel filtration, BALB/c mice, purchased from the above source and bred in our laboratory, were used at more than 3 months of age.

Media and Culture Conditions——Unless otherwise stated, cells were cultured in RPMI 1640 medium (Nissui Seiyaku Co., Japan) supplemented with 100 U/ml of penicillin, 100 μg/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 mice (5×10^6 cells/ml) were cultured with 5 μg/ml of concanavalin A (Con A; Sigma Chemical Co., St. Louis, M.O.) for 48 h. Then 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.

Acid Treatment of Lymphokine Supernatants——The lymphokine supernatants were dialyzed first against either 0.05 M KCl–HCl buffer (pH 2) or 0.01 M phosphate-buffered saline (PBS pH 7.2) for 24 h at 4 °C, and then against serum-free RPMI 1640 medium for an additional 24 h.

Gel Filtration——Spleen cells of BALB/c mice (2×10^7 cells/ml in serum-free RPMI 1640 medium) were cultured with 5 μg/ml of Con A for 48 h as above. Then 40 ml of the supernatant was lyophilized and reconstituted with 4 ml of distilled water; 2.5 ml of the solution was applied to a Sephadex G-100 column (2.2×82.5 cm) equilibrated with 0.01 M PBS (pH 7.2), and eluted with same buffer at the rate of 7.6 ml/h. Fractions of 5 ml were collected, and the activities in each fraction to augment chemiluminescence and cytotoxicity of macrophages were assayed. The column was calibrated with bovine serum albumin (mol. wt. 66000), and cytochrome c (mol. wt. 12400) as marker proteins.

Preparation of Macrophage Activating Factor (MAF)——The details of the preparation of MAF were described elsewhere (in preparation). Briefly, Con A-stimulated BALB/c mouse spleen cell culture supernatants (serum-free) were concentrated by ultrafiltration and lyophilization, and applied to a Sephadex G-100 column. Fractions eluted by 0.01 M PBS (pH 7.2) were assayed by means of cytotoxicity test. The active fractions (mol. wt. 30000—60000) were pooled and termed MAF. The pooled MAF contained less than 0.1 ng/ml endotoxin as determined by using the Limulus amebocyte lysate test (Limulus HS single test Wako, Wako Pure Chemical, Japan).

Preparation of Macrophages and Treatment with Lymphokines——Macrophages were obtained from the peritoneal cavities of mice that had been injected intraperitoneally with 2.5 ml of thioglycollate medium (TGC medium, fluid; Nissui) 4 d earlier. These peritoneal exudate cells were plated on plastic dishes (Lux 5221, 35×10 mm) at a cell density of 2×10^6 cells/dish. After incubation for 1—2 h, nonadherent cells were removed by washing twice, and then the dish-adherent cells, regarded as macrophages, were incubated with 2 ml of media containing lymphokines for various times.

Measurement of Chemiluminescence——In order to simplify the measurement of macrophage chemiluminescence, we previously devised a new type of apparatus. The outstanding features of the apparatus and the method for the measurement of macrophage chemiluminescence using it were described previously.[9] Briefly, a dish with adherent macrophages was washed twice with Hanks' balanced salt solution without phenol red (HBSS) and then placed in the reaction chamber warmed at 37 °C. The chemiluminescence was measured with a Lumicounter 1000 (Niti-On Medical and Physical Instruments Mfg. Co., Japan) equipped with an integrator-timer, immediately after the addition of 1 ml of the chemiluminescent reagent solution. The chemiluminescent reagent solution consisted of 100 μg/ml of luminol (Wako Pure Chemical Ltd., Japan) and 500 ng/ml of phorbol-12-myristate-acetate (PMA; Sigma) in HBSS. The amount of chemiluminescence was expressed as the integrated counts for 5 min, and the chemiluminescence index was calculated as follows.

$$\text{chemiluminescence index} = \frac{\text{chemiluminescence of lymphokine-treated macrophages}}{\text{chemiluminescence of control macrophages}}$$

Cytotoxicity Test——The cytotoxicity test was performed as described previously.[9] TGC-elicited peritoneal

cells were plated on 24-well plates (Falcon 3047) at 1×10^6 cells/well, and then incubated for 1 h. After removal of non-adherent cells, 0.5 ml of medium containing lymphokines was added. At 1—2 h later, 1 ml of a prelabeled FM3A cell suspension (5×10^4 cells/ml) was added to each well. The prelabeled FM3A cells were prepared by incubating FM3A cells for 24 h in a medium containing 0.2 μCi/ml of [^{3}H]thymidine (5 μCi/mmol; RCC Amersham, England). Each well contained lipopolysaccharide (LPS; Sigma, no. L-3129, from *E. coli*, 10 ng/ml) in a final volume of 1.5 ml. After incubation for 48 h, the culture of each well was centrifuged, and 0.5 ml of the supernatant was mixed with 9.5 ml of Univergel II (Nakarai Chemicals, Kyoto), and then the radioactivity was measured with a liquid scintillation counter. The total radioactivity incorporated into the target cells was measured after solubilization of the cells with 0.5% sodium dodecyl sulfate (SDS). Cytotoxicity was estimated by measuring the release of incorporated [^{3}H]thymidine from prelabeled tumor cells in triplicate cultures and expressed as a percentage with respect to the SDS total counts (% lysis) or as specific cytotoxicity according to the following formula;

$$\text{specific cytotoxicity (\%)} = \frac{\text{experimental culture cpm} - \text{control culture cpm}}{\text{total cpm} - \text{control culture cpm}} \times 100$$

In the experiment to examine the time course of the effect of lymphokines on macrophage cytotoxicity, the method was modified as follows. Macrophages were incubated with lymphokines (1 or 1.5 ml) for various times, and washed twice with HBSS to remove the residual lymphokines, then incubated with the target cells in 1.5 ml of media containing 10 ng/ml of LPS. Cytotoxicity was estimated as above after a further 48 h incubation.

Determination of Protein Contents of Macrophages——Dish-adherent macrophages were lysed with 1 ml of 0.5 N NaOH for 1 d, and then the protein contents were determined by Lowry's method using bovine serum albumin as a standard.

Statistical Analysis——The statistical significance of differences was determined by the use of Student's *t*-test.

Results

Effect of Incubation Time with Lymphokines on Macrophage Chemiluminescence

Figure 1 shows the time course of the effect of lymphokines on macrophage chemiluminescence. When macrophages were incubated with 10% lymphokine supernatants, their ability to generate chemiluminescence increased rapidly, reaching a maximal level at 2—4 h, and then decreased to the control level. Such a rapid increase in chemiluminescence of lymphokine-treated macrophages (LTM) has already been reported.[9] However, when the incubation time was further prolonged, the chemiluminescence of LTM began to increase again, and reached about 2-fold the control level after 3 d. Such a biphasic increase in chemiluminescence of LTM was reproducibly observed in repeated experiments.

There was no difference between the protein contents of the macrophages incubated with or without lymphokines for 3 d, while the chemiluminescence generated from LTM was about 2-fold greater than that of the controls (Table I). Therefore the increase in the chemiluminescence of LTM is not due to the increase in the macrophage cell numbers. So, it is likely that the ability to generate chemiluminescence of LTM itself increased biphasically during incubation.

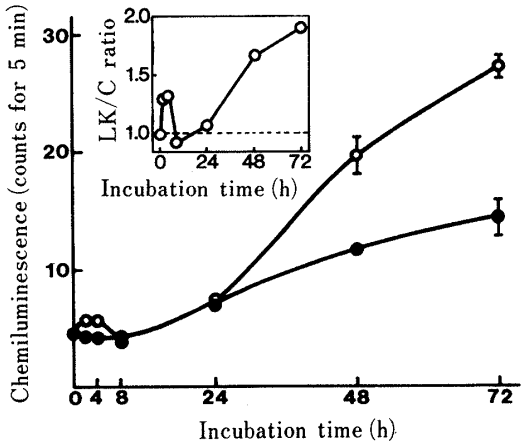

Fig. 1. Effect of Incubation Time with Lymphokines on Macrophage Chemiluminescence

Macrophages were incubated with media containing 10% lymphokine supernatant (○) or 10% control supernatant (●) for various times, and then the chemiluminescence generated from the macrophages was measured. Each point represents the mean ± S.D. of 3 determinations. The chemiluminescence of macrophages incubated with medium alone was almost the same as that of the control. The ratio of the chemiluminescence of LTM to that of the control is shown in the inset.

TABLE I. Comparison of the Ability to Generate Chemiluminescence and the Protein Contents of Macrophages Incubated with Lymphokines for 3 d

Macrophages incubated with	Chemiluminescence (counts for 5 min)	Protein content (μg/dish)	Chemiluminescence/ mg protein
Before incubation	4.5±0.17	173± 7.4	25.7±1.67
Medium	16.1±0.66	361±37.6	44.9±5.45
10% control sup	14.4±1.51	347± 8.2	41.6±3.54
10% LK sup	27.3±1.00[a)]	345± 3.8	79.1±2.99[a)]

Macrophages were incubated for 3 d with or without lymphokines, and then the chemiluminescence and the protein contents of the macrophages were measured. Each value represents the mean±S.D. of 3 determinations. *a*) Significantly different from medium and 10% control sup-treated macrophages ($p<0.001$).

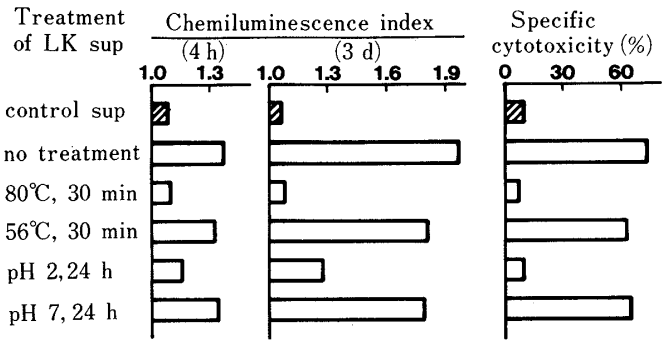

Fig. 2. Effect of Heat and Acid Treatments of Lymphokines on Their Activities to Induce Macrophage Chemiluminescence and Cytotoxicity

Lymphokine supernatants were treated under the indicated conditions, and their activities to induce macrophage chemiluminescence and cytotoxicity were determined at a concentration of 10% as described in Materials and Methods.

Physicochemical Properties of the Lymphokines Affecting the Macrophage Chemiluminescence

Several mechanisms are possible to explain the biphasic increase in chemiluminescence of LTM. For example, there may be two kinds of lymphokines, one causing the early response and the other the late response. To check this possibility, we examined whether the two lymphokines were distinguishable in terms of the physicochemical properties.

Previously, we showed that the early increase in chemiluminescense of LTM was correlated with its cytotoxic activity.[9)] So we first compared the physicochemical properties of the lymphokines which increase the macrophage chemiluminescense with those of interferon-γ, which is a well-known lymphokine inducing macrophage cytotoxicity.[10)]

As shown in Fig. 2, the increasing effects of lymphokine supernatants on the chemiluminescence and cytotoxicity of macrophages were both reduced markedly by heating at 80 °C, but not at 56 °C, for 30 min or by dialysis at pH 2, but not at pH 7, for 24 h. It should be noted that the effects on the early (4 h) and the late (3 d) chemiluminescence were both reduced at the same time.

Thus the lymphokine(s) which augments macrophage chemiluminescence biphasically is acid-labile (pH 2, 24 h), heat-stable (56 °C, 30 min) and nondialyzable. These properties are consistent with those of interferon-γ (type II interferon).[11)]

Gel Filtration

Next we examined whether the two lymphokines supposed to be responsible for the early and the late chemiluminescence were separable by Sephadex G-100 gel filtration. In this section, we designate the lymphokines which increase macrophage chemiluminescence as

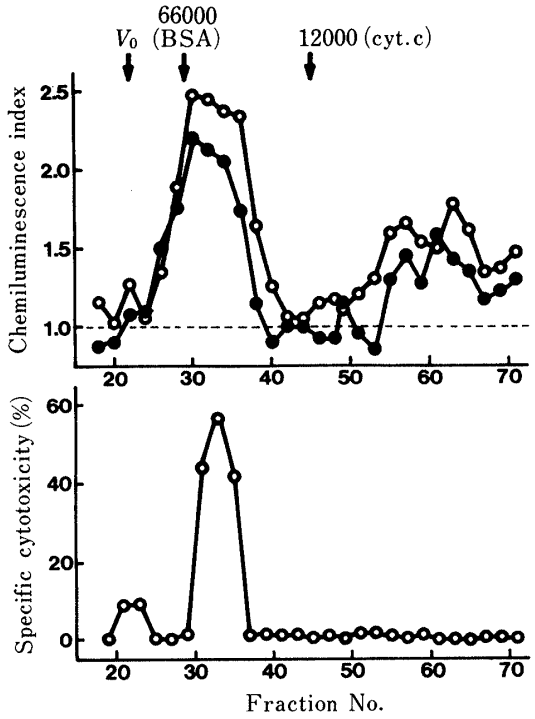

Fig. 3. Fractionation of the Activities to Induce Macrophage Chemiluminescence and Cytotoxicity by Sephadex G-100 Gel Filtration

Serum-free lymphokine supernatants were fractionated by Sephadex G-100 gel filtration, and each fraction was diluted to 10% with medium, then the activities to induce macrophage chemiluminescence and cytotoxicity were determined as described in Materials and Methods. Chemiluminescence was measured after incubation times of 3—5 h (○) and 3 d (●).

chemiluminescence augmenting factor (CAF), and the lymphokines which induce cytotoxicity in macrophages as macrophage activating factor (MAF).

In contrast to the concentrated elution pattern of MAF (molecular weight of 30000—60000), CAF was not only eluted broadly, but also separated into two distinguishable regions intercepted by the retention volume of cytochrome c (Fig. 3). The rapidly eluted major region of CAF corresponded well to that of MAF, while the slowly eluted region of CAF had no activity to induce cytotoxicity. Similar results were obtained in three individual experiments. In the control supernatants, no activity to augment macrophage chemiluminescence was observed in any fraction obtained by Sephadex G-100 gel filtration (data not shown). These results suggest that there are at least two kinds of lymphokines which augment macrophage chemiluminescence, and one of them did not augment cytotoxicity. The latter lymphokine(s) has not yet been characterized in detail.

However, it was impossible to separate the lymphokines into two parts according to the difference in the mode of action on macrophage chemiluminescence by use of Sephadex G-100 gel filtration, since each of the two CAF fractions had both activities to induce the early (4 h) and the late (3 d) chemiluminescence.

Time Course of the Effect of MAF on Macrophage Chemiluminescence and Cytotoxicity

It would be interesting to know whether other cell functions of macrophages are also altered biphasically during incubation with lymphokines. So we examined the effect of incubation time with lymphokines on macrophage cytotoxicity, since the tumoricidal activity is one of the most important indicators of macrophage activation. In this study, partially purified MAF fractions were used as a source of lymphokines.

Figure 4 shows the time course of the effect of MAF on macrophage chemiluminescence and cytotoxicity. In contrast to the clear biphasic increase in chemiluminescence, the increase in cytotoxicity of MAF-treated macrophages was observed only at short incubation times.

At incubation times within one day, the time course of the effect of MAF on macrophage cytotoxicity was well correlated with that on chemiluminescence. That is, the cytotoxic activity of MAF-treated macrophages increased rapidly, reaching a maximal level at 4 h, then

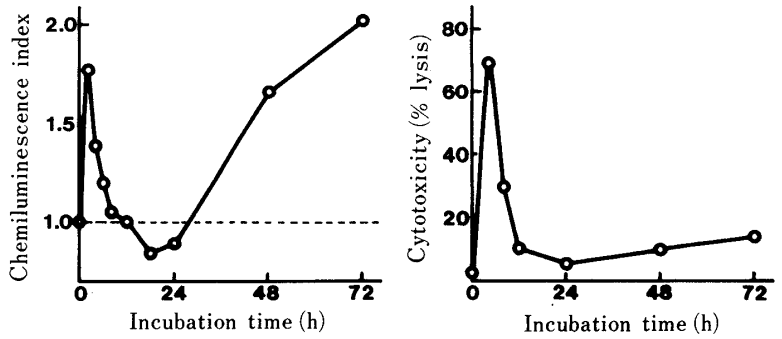

Fig. 4. Time Course of the Effect of Lymphokines on Macrophage Chemiluminescence and Cytotoxicity

Macrophages were incubated with media containing 1% MAF fractions for various times, and then the chemiluminescence and cytotoxicity were assayed as described in Materials and Methods. Each point was calculated from the mean of triplicate cultures.

progressively decreased to the control level, taking a similar course to the chemiluminescence.

When the incubation time was further prolonged, the chemiluminescence of MAF-treated macrophages increased again, while the cytotoxicity did not clearly increase. In some experiments, however, a slight increase in cytotoxicity (specific cytotoxicity, less than 20%) was observed in macrophages incubated with MAF for 3 d (data not shown). However, the increase in cytotoxicity on prolonged treatment with MAF was not only weak as compared with that in the case of short-term treatment, but also its reproducibility was poor. Thus at the present time, it can not be concluded that the cytotoxic activities of MAF-treated macrophages increase biphasically during incubation.

Discussion

Previously, we reported that the effect of lymphokines on macrophage chemiluminescence was short-lived, that is, it became maximum at about 4 h, and then disappeard on further incubation.[9] In that work, the incubation time was limited to within one day. In this report, however, we showed that the increasing effect of lymphokines on macrophage chemiluminescence, having disappeared once, began to appear again when the incubation time was further prolonged. That is, the ability to generate chemiluminescence of LTM increased biphasically during incubation.

The TGC-elicited macrophages seem to grow under our culture conditions, since the protein content increased about 2-fold during 3 d of incubation (Table I). However, there was no difference among the protein contents of macrophages which were incubated with lymphokine supernatants, control supernatants and medium alone. That is, the increase in chemiluminescence of LTM is not due to the increase in macrophage cell numbers.

It has been reported that lipopolysaccharide increased the ability to generate chemiluminescence of macrophages at concentrations of more than 0.1 ng/ml.[12] However, we can rule out the possibility that LPS, which might contaminate the lymphokine preparations, caused the increase in the macrophage chemiluminescence, since even a stock solution of the pooled MAF fractions contained less than 0.1 ng/ml of endotoxin.

Several mechanisms are possible to explain the biphasic increase in chemiluminescence of LTM. For example, it may be that (a) there are two kinds of lymphokines, that is, one causing the early response and the other the late response, (b) there are two subgroups of macrophages, that is, one responding rapidly to the lymphokines and the other slowly, (c) there is a difference in the maturation of macrophages between the early stage and the late stage of the incubation, and so on.

In order to examine the first possibility, we attempted to separate the two lymphokines. Although the two lymphokines were not distinguishable in terms of physicochemical properties, it was revealed that the lymphokines which induce the early (4 h) and the late (3 d) chemiluminescence, as well as MAF, were both acid-labile (pH 2), heat-stable (56°C, 30 min) and nondialyzable. These properties are consistent with those of interferon-γ (type II interferon).[11]

By use of Sephadex G-100 gel filtration, the lymphokines increasing macrophage chemiluminescence were divided into at least two distinguishable regions. The first major region eluted earlier than cytochrome c corresponds to the region of MAF. However, the second minor region eluted later than cytochrome c had no activity to induce cytotoxicity. Nacy *et al.*[13] reported that the lymphokines inducing rickettsicidal activity in macrophages were separated into three distinct regions (mol. wt. of 125000, 45000, and less than 10000) by Sephadex G-200 gel filtration, but that the activity to induce cytotoxicity was observed only at the region of molecular weight 45000. Our results obtained by measuring the macrophage chemiluminescence resemble the results of Nacy *et al.* The molecule(s) eluted later than cytochrome c were supposed to have affinity for Sephadex and not to be low-molecular-weight substances, since the filtrate obtained from the ultrafiltration (cut off mol. wt. 20000) of the lymphokine supernatants had no activity to induce macrophage chemiluminescence (data not shown). However, the detailed characterization of this lymphokine has not yet been done. In any event, we could not separate the two lymphokines responsible for the early and the late chemiluminescent response by use of gel filtration, since both activities were always found together in every active fraction.

The failure to separate the lymphokines into two parts according to the difference in the mode of action on macrophage chemiluminescence suggests that there is a lymphokine which induces a biphasic chemiluminescent response in macrophages by itself. The results from the study on the physicochemical properties of the lymphokines suggest that it may be interferon-γ. Quite recently, the above speculation was confirmed by use of recombinant murine interferon-γ. The details will be reported elsewhere. Hereafter, we intend to investigate the mechanism by which macrophages exhibit the biphasic chemiluminescent response on treatment with a single lymphokine.

In this investigation, we also examined the effect of incubation time with lymphokines on macrophage cytotoxicity in order to know whether the lymphokines alter other cell functions biphasically in a similar manner to macrophage chemiluminescence. Unfortunately, we could not demonstrate a biphasic increase in cytotoxicity of LTM during incubation.

In the early stage of incubation, the time course of the effect of lymphokines on macrophage cytotoxicity was well correlated with that on chemiluminescence. Ruco and Meltzer[14] have also reported that the effect of lymphokines on the induction of cytotoxic macrophages was short-lived. We suggest that the early chemiluminescent response of LTM represents a certain state of activation, *e.g.*, an induction step of cytotoxic activity.

On the other hand, in the late stage of incubation, the cytotoxicity of LTM did not increase so much as compared with the chemiluminescence. However, sometimes a slight increase in cytotoxicity (specific cytotoxicity, less than 20%) was observed in macrophages incubated with lymphokines for 3 d. So we can not say definitely that the cytotoxicity does not increase biphasically during the incubation with lymphokines in every case. It should be noted that, in this investigation, cytotoxic activity of LTM was assessed by measuring the release of [^{3}H]thymidine from prelabeled target tumor cells after a further 48 h incubation with 10 ng/ml LPS, while the chemiluminescence was measured immediately after the treatment with lymphokines. This long incubation for the cytotoxicity test may be a reason why a definite biphasic increase in cytotoxicity was not observed. It may be possible to detect the biphasic increase in cytotoxicity of LTM, if the assay could be completed in a short time as in the case

of the chemiluminescence measurement.

There are other useful markers of macrophage activation, such as antibody-dependent cell-mediated cytotoxicity, bactericidal activity *etc.*, and the mechanisms of these actions are thought to be different from that of the cytotoxicity examined in this report. It remains possible that several kinds of cell functions are altered biphasically during incubation with lymphokines in a similar manner to the chemiluminescence. It is important to know what kinds of cell functions are correlated with the macrophage chemiluminescence, in order to make sense of the biphasic chemiluminescent response of LTM.

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