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Rapid Measurement of Phagocytosis by Macrophages

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A rapid method for measurement of phagocytosis by macrophages was investigated by the use of fluorescein-conjugated zymosan (Fl-zymosan) particles. Adherent peritoneal cells obtained from mice were used as macrophages. The fluorescence intensity of Fl-zymosan measured with a fluorescence spectrophotometer was proportional to the number of particles. Fl-zymosan particles were incubated with macrophages in a flat-bottomed 96-well tissue culture plate at 37 °C in a CO_2-incubator. After the removal of nonphagocytized particles, the fluorescence intensity of particles phagocytized by macrophages was measured with a fluorescence spectrophotometer (microplate reader). In the time course assay, the fluorescence intensity reached a maximum 20 min after the initiation of incubation, and thereafter maintained a constant level till 1 h in both normal and OK-432 injected groups. It was shown that the fluorescence intensity was dependent on the numbers of macrophages and Fl-zymosan particles in both normal and OK-432-injected groups when different numbers of particles (1×10^6, 5×10^6 or 1×10^7) were added to cultures each containing different numbers of macrophages (1×10^5, 2×10^5 or 4×10^5). The macrophages obtained from mice which were injected intraperitoneally (i.p.) with *Propionibacterium acnes* or proteose peptone as well as OK-432 showed higher phagocytic activity. Furthermore, it was also shown that the phagocytic activity measured with a fluorescence spectrophotometer agreed with that measured with a fluorescence microscope. The Fl-zymosan particles were applicable to the phagocytosis assay of opsonized particles.

These results indicate that the method described here is useful for determination of macrophage phagocytic activity.

Keywords——fluorescein-conjugated zymosan; phagocytosis; macrophage; OK-432; opsonin; fluorescence spectrophotometer

Introduction

Phagocytosis is one of the important function of macrophages. The function is known to be modulated by exposure to antigens or biological response modifiers, and is generally assayed as a parameter of the activation of macrophages.[1,2] At the present time, phagocytosis is usually assayed by directly counting the ingested particles (*e.g.* latex beads, bacterial cells and zymosan particles) or measuring an isotope ingested with particles (*e.g.* isotope-labeled bacterial cells and red blood cells).[2,3] However, these procedures are time-consuming. A recent report by Oda and Maeda[4] described a fluorometric assay method for phagocytosis by macrophages and polymorphonuclear leukocytes by utilizing fluorescent particles. The method is sensitive and simple, and therefore many assays can be done in a short time. In the assay procedures, however, a relatively large number of phagocytic cells were required. Therefore, it may be still inconvenient to assess the function of phagocytic cells which are not readily available in large amounts.

In the present study, we attempted to improve the method reported by Oda and Maeda.[4] This study focused on the phagocytosis of macrophages, and the assays were performed on a small scale (in a 96-well tissue culture plate). Two kinds of macrophages, taken from normal

and stimulant (OK-432, *Propionibacterium acnes* and proteose peptone)-injected mice, were used and their phagocytic activities were compared under several assay conditions.

Materials and Methods

Preparation of Fluorescein-Conjugated Zymosan (Fl-Zymosan)——Fl-zymosan was prepared according to the method of Glabe *et al.*[5] with a slight modification. Briefly, 200 μg of zymosan (Zymosan A; Sigma Chemical Co., Saint Louis, Mo.,) suspended in 20 ml of water was boiled for 1.5 h, then 100 μg of BrCN was added to the suspension. The mixture was adjusted to pH 11 and maintained at that pH for 5 min by addition of 0.2 N NaOH. The activated zymosan was resuspended in 40 ml of 0.2 M sodium borate buffer (pH 8.0) solution and reacted with 20 mg of fluoresceinamin (Fluka AG, Buchs), After incubation for 5 min, Fl-zymosan was separated from the unreacted fluoresceinamin by centrifugation. Fl-zymosan thus prepared was suspended in distilled water and sonicated (50 W, 20 KHz for 1 min) by an ultrasonicator (Ohtake Works, Tokyo). Fluoresceinamin dissociated from the conjugate was removed by centrifugation (750 × *g* for 5 min). The precipitate was resuspended in RPMI 1640 medium (Nissui Seiyaku Co., Ltd., Tokyo) and centrifuged (50 × *g* for 1 min) to remove aggregated Fl-zymosan. The number and size of Fl-zymosan particles in the supernatant were determined by a Coulter counter (Coulter Electronics, Inc., Hialeah, Fla.). The sizes of particles were between 0.5 and 1.5 μm.

Preparation of Peritoneal Exudate Cells——Male 6—8 weeks old ICR mice were purchased from Shizuoka Agricultural Cooperative Association for Laboratory Animals, Shizuoka. Normal mice and stimulant-injected mice were used as sources of peritoneal cells. OK-432 (a group A streptococcal preparation, kindly provided by Chugai Pharmaceutical Co., Ltd., Tokyo, 1KE/mouse), *P. acnes* (kindly provided by Kowa Co., Ltd., Tokyo, 400 μg/mouse) and proteose peptone (Difco Laboratories, Detroit, Mich.) were injected i.p. as the stimulants into mice. The peritoneal cavities of mice were washed twice with 5 ml of Hanks' balanced salt solution (HBSS). The cells collected were washed twice and resuspended in RPMI 1640 medium.

Phagocytosis Assay——In most experiments, Fl-zymosan particles in RPMI 1640 medium were used. Fl-zymosan particles opsonized with complement were used in one experiment. The opsonization was performed as follows: Fl-zymosan particles (1×10^4) were incubated with 1 ml of guinea pig serum for 30 min at 37 °C. The particles were washed twice with HBSS by centrifugation and resuspended in RPMI 1640 medium. These particles were added to the adherent cell culture. After incubation in a CO_2-incubator for various times at 37 °C, the adherent cells were washed twice with RPMI 1640 medium to remove any nonphagocytized zymosan particles. The adherent cells were then solubilized by addition of 100 μl of 50 mM sodium cholate. The fluorescence intensity was measured with a fluorescence spectrophotometer (microplate reader MTP-32; Corona Electric Co., Ltd., Tokyo) with an excitation wavelength of 490 nm and an emission wavelength of 530 nm. In one experiment, the number of Fl-zymosan particles phagocytized was counted by utilizing a fluorescence microscope (FLUOPHOT VFS-R; Nihon Kogaku Kogyo Co., Ltd., Tokyo).

Results and Discussion

First, the relationship between the number of zymosan particles and fluorescence intensity was examined. The intensity of 5×10^4, 1×10^5, 5×10^5, 1×10^6, 5×10^6, 1×10^7, 5×10^7 or 1×10^8 zymosan particles/ml was determined. As shown in Fig. 1, the fluorescence intensities increased proportionally to the number of zymosan particles. This result indicates that the number of zymosan particles can be calculated from the fluorescence intensity at least in the range from 5×10^4 to 1×10^8 zymosan particles/ml.

Next, the time-course of phagocytosis by macrophages was examined. The peritoneal macrophages (5×10^5) obtained from normal or OK-432-injected mice were incubated with 1×10^6 zymosan particles, and the fluorescence intensities in macrophages were determined 10, 20, 30 and 60 min after the initiation of incubation. As shown in Fig. 2, the fluorescence intensity reached a maximum at 20 min and thereafter maintained a constant level till 60 min in both normal and OK-432-injected groups. However, the fluorescence intensity observed in the OK-432 (an agent for activation of macrophage function[6])-injected group was markedly higher than that in the normal group.

In order to determine the correlation between the numbers of macrophages and zymosan particles, 1×10^6, 5×10^6 or 1×10^7 zymosan particles were added to the culture containing 1×10^5, 2×10^5 or 4×10^5 macrophages, and then the fluorescence intensity in macrophages

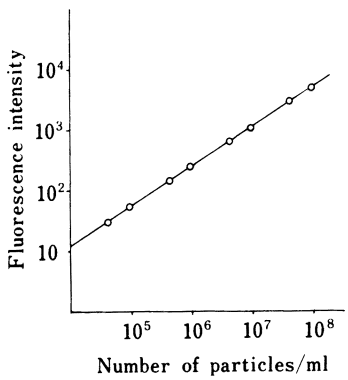

Fig. 1. Relationship between Fluorescence Intensity and Fl-Zymosan Particle Concentration

Each point is the mean of triplicate values.

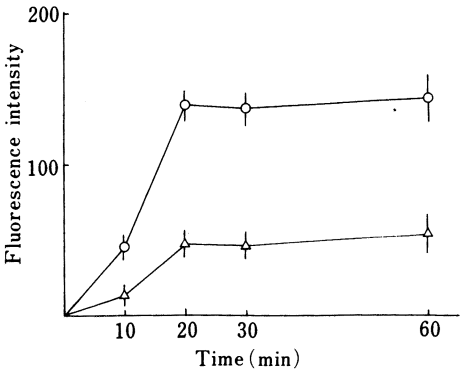

Fig. 2. Time Course of Phagocytosis of Fl-Zymosan Particles by Macrophages

The peritoneal macrophages (5×10^5) obtained from normal (△) or OK-432 (○)-injected mice were incubated with 1×10^6 Fl-zymosan particles at 37 °C, and the fluorescence intensity in the macrophages was determined.

was determined 20 min after the initiation of culture. The results are shown in Fig. 3. The fluorescence intensity was dependent on the number of macrophages in both macrophage groups. Moreover, the fluorescence intensities increased in a dose-dependent manner with respect to the number of zymosan particles.

The phagocytic activities of macrophages obtained from mice which had been injected i.p. with OK-432, *P. acnes* and proteose peptone were compared. Fl-zymosan particles (1×10^6) were incubated with 5×10^5 or macrophages taken from several groups of mice for 20 min, and the fluorescence intensity of phagocytized Fl-zymosan particles in each macrophage group was determined. As shown in Table I, all of the macrophages taken from stimulant-injected mice showed higher phagocytic activities than macrophages from normal mice, and the stimulation indices were between 1.5 and 2.4.

It is known that the fluorescence intensity of fluorescein changes depending on the pH. In the case of Fl-zymosan, the fluorescence intensities at several pHs (buffered with 0.1 M citrate buffer) were as follows; 178 (±2) at pH 4.0, 388 (±15) at pH 5.0, 1,169 (±117) at pH 5.5, and 1,302 (±66) at pH 6.0 when 1×10^7 Fl-zymosan particles were used for the determination. On the other hand, it was reported that the pH of vacuoles in macrophages changed during the phagocytosis of yeast.[7] Therefore, there was a possibility that the strong fluorescence intensities observed in the macrophages derived from stimulant injected mice (Table I) arose from the change of intravacuolar pH in macrophages. In order to rule out this possibility, we determined the pH of solutions after the solubilization of macrophages. It was found that all of the macrophage lysates tested showed the same pH (pH 7.5) as sodium cholate solution used for the solubilization, even when sodium cholate dissolved in citrate buffer (0.1 M, pH 6.5) was used as an agent for the solubilization. These results indicated that the pH of macrophage vacuoles did not affect the fluorescence intensity in this system.

The amount of Fl-zymosan particles ingested by macrophages determined with a fluorescence spectrophotometer was compared with that determined with a fluorescence microscope. Fl-zymosan particles (1×10^7) were incubated with 5×10^5 macrophages taken from normal or OK-432-injected mice. The fluorescence intensities in the normal and OK-432 group were 322 (±8) and 604 (±36), respectively. These intensities correspond to 38.7 or 72.6%, respectively, when the fluorescence intensity (832) or total Fl-zymosan particles (1×10^7) is considered as 100%. The numbers of Fl-zymosan particles ingested into the

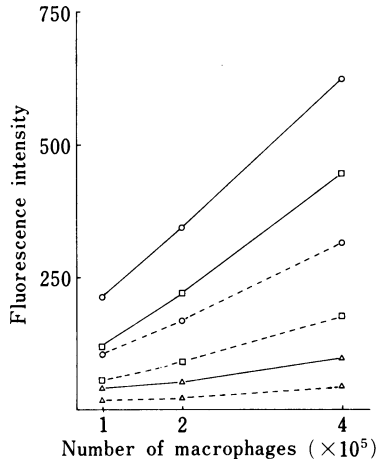

Fig. 3. Phagocytosis Assay Using Different Number of Macrophages and Fl-Zymosan Particles

Macrophages were obtained from normal (----) or OK-432-injected (——) mice, and 1×10^6 (△), 5×10^6 (□), or 1×10^7 (○) zymosan particles were added to cultures containing 1×10^5, 2×10^5, or 4×10^5 macrophages. The fluorescence intensity in the macrophages was determined.

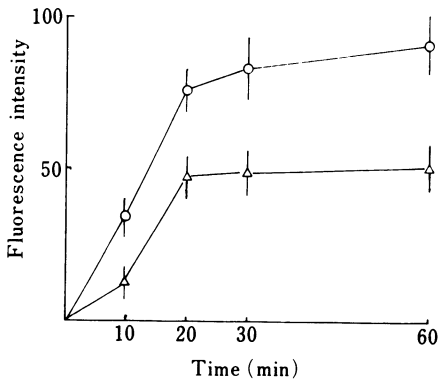

Fig. 4. Time Course of Phagocytosis of Unopsonized and Opsonized Fl-Zymosan Particles by Macrophages

Peritoneal macrophages (5×10^5) obtained from normal mice were incubated with 1×10^6 unopsonized (△) or opsonized (○) Fl-zymosan particles at 37 °C, and the fluorescence intensity in the macrophages was determined.

TABLE I. Phagocytic Activities of Stimulant-Induced Macrophages

Stimulant[a]	Dose/mouse	Fluorescence intensity[b]
Nil		471±36
OK-432	1 KE	1121±75[c]
P. acnes	400 μg	958±82[c]
Proteose peptone	100 μg	703±57[c]

a) Macrophages were taken from mice which had been injected with each stimulant 4 d after the injection. *b*) Each value is the mean±standard deviation. *c*) Significance (*p* value) of the difference between the Nil and treated groups was evaluated according to Student's *t*-test. $p < 0.01$.

TABLE II. Microscopic Evaluation of Phagocytosis of Fl-Zymosan Particles

No. of particles[a]	% phagocytosis[b]	
	Normal group	OK-432-injected group
0—1	35±6	5± 3
2—4	39±8	16± 5
5—7	24±5	69±11
8—	2±2	10± 4

a) The number of Fl-zymosan particles ingested by a macrophage. *b*) After 20 min of incubation at 37 °C, more than 200 macrophages per group were counted under a fluorescence microscope. Values are mean±standard deviation.

macrophages determined by a fluorescence microscope are shown in Table II. In the normal group, macrophages which had ingested small numbers of particles were in the majority. On the other hand, macrophages which had ingested 5—7 particles or more amounted to about 70% in the OK-432 group. The total number of particles ingested was calculated to be 297 for the normal group or 555 for the OK-432 group, and the ratio of these numbers (555/297) is 1.9. This ratio corresponds well to that calculated from the fluorescence intensities (72.6/38.7) determined with a fluorescence spectrophotometer.

Phagocytic response of macrophages to opsonized Fl-zymosan was compared with that to unopsonized particles. Fl-zymosan particles were opsonized by incubation with guinea pig serum. The treatment did not affect the fluorescence intensity of Fl-zymosan. The opsonized or unopsonized Fl-zymosan particles (1×10^6) were added to a macrophage (5×10^5) culture, and the fluorescence intensity in macrophages was determined. In both systems, the fluorescence intensity reached a maximum at 20 min and thereafter maintained a constant

level till 60 min (Fig. 4). However, the intensity in the opsonized group was about twice that in the unopsonized group at each incubation time.

Oda and Maeda[4] examined phagocytic activities of macrophages and polymorphonuclear leukocytes by utilizing fluorescein-conjugated particles including bacterial and yeast cells, and latex particles. In the assay, 1×10^6 phagocytic cells were placed in 16 mm wells (24-well tissue culture plate) and incubated with the particles in a 5% CO_2-incubator. It was found that the rate of ingestion of particles at 37 °C by these phagocytic cells was essentially linear with time up to 60 min, and then reached a plateau after 120 min.

In the present study, we have adapted the technique Oda and Maeda[4] to a smaller scale for the examination of adherent macrophages. By this method, it was possible to measure the phagocytic activity of even when a small number of macrophages ($1—2 \times 10^5$).

It was also found that 1) the time necessary to reach maximum fluorescence intensity after the initiation of phagocytosis was short (20 min), and 2) opsonized F1-zymosan particles were applicable instead of unopsonized particles. The method reported here cannot distinguish F1-zymosan particles associated with macrophages or phagocytized. Therefore, there was a possibility that the fluorescence intensity detected after the phagocytosis assay was partly due to cell-associated F1-zymosan particles. In the present experiments, however, the macrophages taken from OK-432 (a macrophage activator)-injected mice exhibited higher phagocytic activities than those of macrophages from normal mice. Additionally, the ratio of activities between normal and OK-432-treated macrophages measured with a fluorescence spectrophotometer agreed well with that measured with a fluorescence microscope.

It is possible that fluorescein may be released from F1-zymosan particles by the action of lysosomal enzymes. However, the culture medium, including nonphagocytized F1-zymosan particles and the metabolites from macrophages, was removed by washing after the completion of incubation. This procedure and the result obtained from the microscopic observation described above are considered to rule out significant release of fluorescein.

Thus, the method presented here is applicable for the rapid determination of macrophage phagocytosis. The use of a microplate reader to measure fluorescence intensity will enhance the usefulness of the method. Zymosan particles seem to be suitable for the present purpose because 1) zymosan itself and F1-zymosan are stable materials, 2) these particles can be used in both unopsonized and opsonized forms and 3) the mechanism of phagocytosis of zymosan particles has been studied specifically[8,9] in addition to general studies on phagocytic mechanisms. Other fluorescent particles such as fluorescent latex and fluorescent bacterial cells may also be applicable, depending on the purposes of studies.

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