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Research Paper

Stimulation of Phagocytic Activity of Alveolar Macrophages Toward Artificial Microspheres by Infection with Mycobacteria

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Purpose. The purpose of this study is to know the effect of uptake of mycobacteria on the phagocytic activity of alveolar macrophage (Mϕ) cells toward poly(lactic-co-glycolic) acid (PLGA) microspheres (MS) loaded with the anti-tuberculosis agent rifampicin (RFP-PLGA MS).
Materials and Methods. Biological functions such as phagocytic activity toward PLGA MS loaded with fluorescent coumarin (cPLGA MS) and toward polystyrene latex MS (PSL MS), and generation of tumor necrosis factor-α (TNF-α) and nitric oxide (NO) were examined using alveolar Mϕ cell NR8383 after they had phagocytosed *Mycobacterium bovis* Calmette-Guérin (BCG), heat-killed BCG (h-kBCG) or *Escherichia coli*.
Results. The ingestion of BCG, h-kBCG, and *E. coli* did not affect the viability of the Mϕ cells within 2 days. The phagocytosis caused generation of TNF-α and NO, being more significant with *E. coli* than with both types of BCGs. The phagocytosis of both types of BCGs stimulated the phagocytic uptake of cPLGA and PSL MS's, which took place prior to the generation of TNF-α or NO, but that of *E. coli* suppressed the uptake of both MS's.
Conclusion. Mycobacterial infection stimulated the phagocytic uptake toward cPLGA MS. These results suggest that RFP-PLGA MS is favorable for overcoming tuberculosis.

KEY WORDS: alveolar macrophage; phagocytosis; polymer microspheres; pulmonary delivery; tuberculosis.

INTRODUCTION

Alveolar macrophage (Mϕ) cells phagocytose invaders, such as pathogenic microorganisms, during respiration to defend the host, which action is the first step of sequential immune reactions. Although most microorganisms are digested by lysosomal enzymes in Mϕ cells (1,2), *Mycobacterium tuberculosis* (MTB) escapes from this immune system by inhibiting the fusion of lysosomes with the phagosomes, and proliferates by using Mϕ cells as incubators (3,4). Hence, inhalation delivery of anti-tuberculosis agents, such as rifampicin (RFP), into the MTB-infected alveolar Mϕ cells is expected to be very effective to overcome tuberculosis. Accordingly, we formulated microspheres (MS) using poly(lactic-co-glycolic) acid (PLGA) as a base, into which RFP was incorporated. The MS thus prepared (RFP-PLGA MS) were well phagocytosed by alveolar Mϕ cells (5), and RFP incorporated into PLGA MS showed potent bactericidal effect on *Mycobacterium bovis* Calmette-Guérin (BCG), which had been taken up by Mϕ cells, the effect being about 100 times greater than that of RFP added in solution form (6).

Our strategy depends on the efficiency of the phagocytic uptake of RFP-PLGA MS by MTB-infected alveolar Mϕ cells. However, it is not clear at present what effects mycobacterial infection exerts on the phagocytic activity of alveolar Mϕ cells toward PLGA MS. It has been reported that infection with mycobacterium species or addition of mycobacterial components, such as tuberculin, activates Mϕ cells by generating cytokines such as tumour necrosis factor-α (TNF-α) and nitric oxide (NO) leading to immune responses against mycobacteria (7–11), although the generation of cytokines is not always associated with the production of NO (12,13). It is reported that TNF-α and NO generated in

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ABBREVIATIONS: BCG, *Mycobacterium bovis* Calmette-Guérin; h-kBCG, heat-killed BCG; CFU, colony-forming units; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; Mϕ, macrophage; MOI, multiplicity of infection; MTB, *Mycobacterium tuberculosis*; MS, microspheres; NO, nitric oxide; PBS, phosphate-buffered saline; PLGA, poly(lactic-co-glycolic) acid; cPLGA MS, PLGA MS loaded with coumarin 6; RFP-PLGA MS, PLGA MS loaded with rifampicin; PSL MS, polystyrene latex MS; RFP, rifampicin; TNF-α, tumor necrosis factor-α; WST-8, 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium, monosodium salt.

the Mϕ cells suppress the phagocytic activity and cause apoptosis, respectively (14,15). Hence, it is of importance to know the effect of MTB infection on the phagocytic activity of Mϕ cells toward polymer microspheres, especially in terms of generation of TNF-α and NO. The effects of MTB-infection on Mϕ cells, however, do not seem to have been examined rigorously in terms of the phagocytic activity of Mϕ cells.

In this study, we determined the effects of MTB infection and those of TNF-α and NO generated on the phagocytic activity of alveolar Mϕ cells toward artificial microspheres PLGA MS loaded with the fluorescent coumarin 6 (cPLGA MS) or RFP (RFP-PLGA MS) and polystyrene latex MS (PSL MS). We used rat alveolar Mϕ cell line NR8383, and *Mycobacterium bovis* Calmette-Guérin (BCG) as models of alveolar Mϕ cell and MTB, respectively. These effects were analyzed in comparison with those of phagocytosis of heat-killed BCG (h-kBCG) and *Escherichia coli* (*E. coli*) which is well known to stimulate Mϕ cells to exert inflammatory responses (16).

MATERIALS AND METHODS

Materials

Sources of materials and reagents used in this study were as follows: PLGA with molecular weight of 10,000 consisting of a monomer composition of lactide/glycolide=75:25 (PLGA7510), referred to as PLGA in this paper unless otherwise noted, from Wako Pure Chemical Industry (Osaka); F-12K medium, from Invitrogen (Carlsbad); Mycobroth, from Kyokuto Pharmaceutical Industrial Co., Ltd. (Tokyo); Fluoresbrite® YG Carboxylate Microspheres 3.00 μm (PSL MS) and coumarin 6, from Polysciences, Inc. (Warrington); 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium, monosodium salt (WST-8), from Dojindo Laboratories (Kumamoto); Dulbecco's phosphate-buffered saline, from Nissui Pharmaceutical Co., Ltd. (Tokyo); Bacto tryptone and Bacto yeast extract, from Becton Dickinson (Franklin Lakes); ampicillin, from Meiji Seika (Tokyo); Percoll, from Sigma-Aldrich (St. Louis); Ziehl-Neelsen carbol-fuchsin stain solution, from Nacalai Tesque, Inc. (Kyoto); and Entellan Neu, from Merck (Darmstadt). Enzyme-linked immunosorbent assay (ELISA) kits were obtained from BioSource International, Inc. (Camarillo). All other chemicals were of the highest grade commercially available.

Alveolar Mϕ Cells

NR8383 cells derived from rat alveolar Mϕ cells (CRL-2192, ATCC, Rockville), which are adherent but easily migrate toward microbes and synthetic MS, were used as Mϕ cells, and were cultured in F-12K medium supplemented with 15% heat-inactivated fetal bovine serum (FBS) and 60 μg/ml ampicillin at 37°C under a stream of 5% CO_2. During the incubation of phagocytosis, the FBS concentration in the medium was maintained to be 4% to keep the number of Mϕ cells constant, and this FBS concentration was used in all the examinations on phagocytic activities of Mϕ cells. The viability and phagocytic activity of Mϕ cells were not affected by this FBS concentration.

Microorganisms

Lyophilized BCG, purchased from Japan BCG group (Tokyo), were suspended in Mycobroth (Kyokuto) and incubated at 37°C with vigorous mixing once a day until the optical density (O.D.) reached the range between 0.2 and 0.8. The concentration of live BCG was determined spectrophotometrically by measuring the O.D. at 600 nm according to the relation between O.D. and colony-forming units (CFUs) of BCG on 2% Vite Medium (Kyokuto) determined by us (1 O.D.=4.1×10^8 cells/ml). For preparing heat-killed BCG (h-kBCG), live BCG cells were boiled at 100°C for 10 min. As the BCG aggregated by boiling, the microbial suspension was centrifuged at 200×*g* for 5 min at room temperature, and the dispersed h-kBCG cells in the supernatant were collected. Then, the Mycobroth suspension containing live BCG or h-kBCG was replaced with phosphate-buffered saline (PBS) containing 0.05% Tween 80 (MP Biomedicals, LLC, Irvine) by centrifuging the cells at 1,700×*g* for 5 min at room temperature, removing the supernatant, and resuspending the microbes in the PBS. The concentrations of both types of BCGs were confirmed by counting the cell number in a light microscopic field.

E. coli (JM109 strain) were grown at 37°C with shaking in LB broth, which contained 1% (*w*/*v*) bacto tryptone, 0.5% (*w*/*v*) bacto yeast extract and 0.5% (*w*/*v*) sodium chloride, until the O.D. reached between 0.2 and 0.8. The concentration of *E. coli* was determined spectrophotometrically at 600 nm by using the relation of 1 O.D.=9.6×10^8 cells/ml [determined from the relation of O.D. to CFUs of *E. coli* established by using LB agar plates containing 1% (*w*/*v*) bacto tryptone, 0.5% (*w*/*v*) bacto yeast extract, 0.5% (*w*/*v*) sodium chloride and 1.5% (*w*/*v*) agar]. Then, LB broth, in which *E. coli* had been suspended, was replaced with PBS as described above for BCG, after which the *E. coli* was killed by 1-day UV irradiation. For viability assays and flow cytometric analysis, *E. coli* (K-12 strain) labeled with Texas Red purchased from Invitrogen (Carlsbad) were used.

Preparation of PLGA Microspheres Containing Coumarin 6

The PLGA MS loaded with the fluorescent coumarin 6 (cPLGA MS) were prepared by the method described previously (5), with some modifications (17). Briefly, PLGA (90 mg) and coumarin 6 (50 μg) were dissolved in chloroform (3 ml) as an oil phase, and the solution was pressed out into a 2.0% (*w*/*v*) polyvinyl alcohol (PVA) aqueous solution through a Shirasu porous glass membrane with a pore size of 1.00 μm. Then, the cPLGA MS were stabilized as an emulsion in 1.0% (*w*/*v*) PVA solution. The volume-averaged diameter of cPLGA MS prepared was 2.64±0.77 μm, as determined with a light scattering particle sizer, Mastersizer 2000 (Malvern Instruments Ltd., Malvern). The PLGA MS loaded with the RFP (RFP-PLGA MS) were prepared using a spray dry method described previously (18). The average diameter of RFP-PLGA MS prepared was about 2.00 μm and the size distribution of those was narrow. The cPLGA MS

and RFP-PLGA MS prepared were regarded to be free from endotoxin, because the phagocytosis of RFP-PLGA MS did not generate TNF-α and NO appreciably.

Determination of Phagocytic Activity Toward BCGs and Synthetic MS by Observation Under a Light Microscope

NR8383 cells were seeded in the incubation medium in a 96-well plate (Becton Dickinson) at a density of 1×10^5 cells/200 μl per well. The Mϕ cells were incubated for various periods at 37°C under an atmosphere of 5% CO_2 after the addition of live BCG or h-kBCG at a multiplicity of infection (MOI) of 10 (10 bacilli per Mϕ). To separate BCGs that had not been phagocytosed from those that had been, the cell suspension was centrifuged at 200×*g* for 5 min at room temperature, and then the supernatant was discarded before the Mϕ cells were resuspended in fresh PBS. These procedures were repeated again; and then an aliquot of the suspension of the Mϕ cells was sequentially mounted on a glass slide, dried completely, and fixed with 10% isotonic formalin. BCGs taken up by Mϕ cells were stained with Ziehl-Neelsen carbol-fuchsin stain solution for 10 min and then destained with 1% HCl–70% ethanol for 5 min. Next, the Mϕ cells were stained for 30 s with ×10 diluent of Loeffler's methylene blue solution, prepared by dissolving 1.5 g methylene blue in 30 ml ethanol before the addition of 100 ml of 0.01% (*w*/*v*) potassium hydroxide. Both types of BCGs phagocytosed by 200 Mϕ cells for each were counted by observation under a light microscope, BH-2 (Olympus, Tokyo) at a magnification of ×1,000. Uptake of RFP-PLGA MS by Mϕ cells were examined essentially by the methods reported previously (19,20).

Flow Cytometric Analysis of Phagocytosis Toward *E. Coli*

NR8383 cells in the incubation medium were seeded in a 96-well plate at a density of 1×10^5 cells/200 μl per well. *E. coli* cells labeled with Texas Red were added at an MOI of 10, and the Mϕ cells were incubated for various periods at 37°C under an atmosphere of 5% CO_2. To separate the *E. coli* that had not been phagocytosed from the Mϕ cells, the cell suspension was centrifuged at 200×*g* for 5 min at room temperature, and the supernatant was discarded. Then, the cells were washed twice with PBS by centrifugation at 200×*g* for 5 min at room temperature; and the supernatant was removed by aspiration between washes. Phagocytic Mϕ cells were determined by using a flow cytometer, FACSAria (Becton Dickinson), to analyze approx. 10,000 Mϕ cells.

Determination of Viability of Alveolar Mϕ's

NR8383 cells (5×10^5 cells/ml per well) in the incubation medium were mixed with either live BCG, h-kBCG or *E. coli* in a 24-well plate (Becton Dickinson) at an MOI of 10 for 6 h at 37°C under an atmosphere of 5% CO_2. Unphagocytosed microbes were separated from the Mϕ cells by two serial washing procedures: centrifuging the cell suspension at 200×*g* for 5 min at room temperature, aspirating the supernatant thoroughly, and resuspending the Mϕ pellet in fresh PBS. Then, the Mϕ cells were resuspended in the medium and further incubated for 1, 2 or 4 day at a density of 5×10^4 cells/100 μl per well in a 96-well plate. WST-8 reagent solution (10 μl) was added to each well containing 100 μl of cell suspension, and then the mixture was incubated for 2 h at 37°C under an atmosphere of 5% CO_2. The WST-8 reagent added was reduced by cellular dehydrogenases derived from living cells to give a yellow colour due to the production of formazan, the concentration of which is known to be proportional to the number of living cells (21). Absorbance of formazan in cell suspension was measured in a spectrophotometer, model 680 (Bio-Rad Laboratories, Hercules), at 450 nm by taking the absorbance at 630 nm as a reference. Viability of NR8383 cells was determined from the absorbance due to formazan, taking the absorbance of the Mϕ cells without incubation with microbes as a reference (100%). The absorbance of formazan in the suspension of live BCG at 5×10^5 cells/100 μl was not changed at all for 2 h, suggesting that WST-8 is not metabolized by live BCG and that it does not affect viability of BCG.

TNF-α and Nitrite Assay

NR8383 cells (1×10^5 cells/200 μl per well) in the incubation medium were incubated with either live BCG, h-kBCG or *E. coli* in a 96-well plate at MOI of 10 for various periods at 37°C under 5% CO_2. Then, cell culture supernatants were collected and stored at −80°C before the assaying of TNF-α and nitrite. The concentration of TNF-α in the thawed supernatants was determined with an ELISA kit (BioSource International, Inc.); and that of nitrite, by the Griess assay. Briefly, 50 μl of Mϕ cell culture supernatant was incubated with an equal volume of the Griess reagent consisting of 1% (*w*/*v*) sulfanilamide, 0.1% (*w*/*v*) *N*-(1-naphthyl)-ethylenediamine dihydrochloride, and 2.5% (*v*/*v*) H_3PO_4 for 10 min at room temperature in a 96-well plate. Then, the absorbance was measured at 550 nm. $NaNO_2$ was used as a reference.

Effects of BCGs and *E. Coli* on the Phagocytosis of cPLGA and PSL MS

NR8383 cells (5×10^5 cells/ml per well) in the incubation medium were incubated with either BCG, h-kBCG or *E. coli* in a 24-well plate at an MOI of 10 for 6 h at 37°C under 5% CO_2. The microbes that had not been phagocytosed were separated from the Mϕ cells by the serial washing procedures described above. The Mϕ cells were further incubated in fresh incubation medium for various periods, and then they were incubated with cPLGA MS or fluorescent-labeled PSL MS at an MS/Mϕ of 10 for 2 h at 37°C under the atmosphere of 5% CO_2. After phagocytosis, extracellular microspheres adhering to the surface of Mϕ cells were dissociated from them by soaking the cells in an acidic buffer solution consisting of 28 mM sodium acetate and 117 mM sodium chloride, the pH of which was adjusted to 4 with 1 M HCl (22); and the Mϕ cells were subsequently separated by centrifugation through a Percoll layer, as described previously (5). Changes in the phagocytic activity of Mϕ cells toward cPLGA or PSL MS caused by phagocytosis of live BCG, h-kBCG or *E. coli* were determined by using the flow cytometer

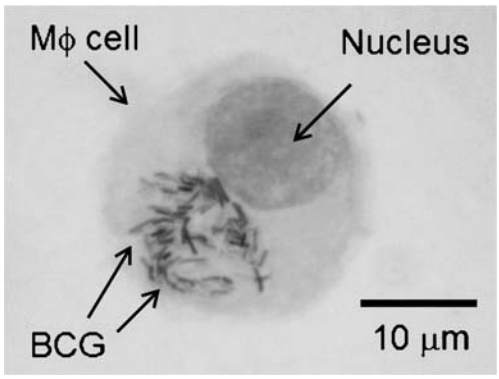

Fig. 1. Microscopic observation of an NR8383 cell incubated with live BCG. After incubation of NR8383 cells with live BCG at an MOI of 10 for 6 h at 37°C, BCG and Mϕ cell were stained with Ziehl-Neelsen carbol-fuchsin stain solution and ×10 diluent of Loeffler's methylene blue solution, respectively; and the Mϕ cell was observed under a light microscope at magnification ×1,000.

(FACSAria, Becton Dickinson) to analyze approx. 10,000 Mϕ cells.

Statistics

Two-tailed Student's *t* tests were performed on original data to assess statistical differences between groups. Significance levels were set at $P<0.05$.

RESULTS

Phagocytic Activity of Alveolar Mϕ Cells Toward BCGs and *E. Coli*

A representative microscopic image of a single Mϕ cell that had taken up live BCG is shown in Fig. 1. Phagocytic activities of NR8383 cells were expressed as the relative population (%) of the Mϕ cells that had phagocytosed these BCGs per a total of 200 Mϕ cells. The time-course of the phagocytic activity of the Mϕ cells toward live BCG and h-kBCG at an MOI of 10 is summarized in Table I. Phagocytosis toward these BCGs proceeded in a similar manner, attaining a saturation level of approx. 30% after incubation for 6 h. It is noteworthy that about 70% of the Mϕ cells did not phagocytose either type of BCG even after 10 h.

The mean number of the BCGs phagocytosed per Mϕ cell that had taken up the bacteria is also summarized in Table I. These results show that Mϕ cells phagocytosed about two bacteria of either type of BCG by incubation for 2 h. The number phagocytosed increased only slightly by further incubation for 2 h (total 4 h), and then almost leveled off after prolonged incubation, suggesting that the capacity of Mϕ cells to take up either type of BCG was about three on average.

Next, we examined the phagocytic activity of NR8383 cells toward *E. coli* to compare it with that toward the BCGs. The phagocytic activity of approx. 10,000 Mϕ cells was determined with a flow cytometer, because determination by optical microscopic observation was difficult due to shrinkage of *E. coli* upon phagocytosis. As shown in Fig. 2, the phagocytic Mϕ cells were well separated from Mϕ cells that had not phagocytosed *E. coli,* as evidenced by the dot–plot profiles and the histograms. Thus, the population of phagocytic Mϕ cells could be determined from the histograms. As shown in Fig. 3, the phagocytosis proceeded time-dependently; and it seemed to attain the maximal level after incubation for about 10 hr. The population of phagocytic Mϕ cells toward *E. coli* at 10 h was about 63%, being more than twice that toward either type of BCG.

Effect of Phagocytosis of Microbes on Viability of Mϕ Cells

As shown in Fig. 4, Mϕ cell viability was not affected at all by the phagocytosis of any of these three types of microbes within 2 days after termination of phagocytosis, but it did decrease considerably on day 4. After incubation for 4 days, the phagocytosis of *E. coli* caused death of almost all the Mϕ cells, and that of live BCG decreased the viability to be 35%. However, approx. 70% of the Mϕ cells were still alive after the phagocytosis of h-kBCG. The increase in the viability to a certain extent observed in the early stage of incubation (on day 1 or 2) could have been due to the mitogenesis by microorganisms (23,24). These results show that NR8383 cells treated with microbes preserved their biological activities for at least 2 days. Hence, we examined hereafter the effects of phagocytosis of microbes on the biological activities of Mϕ cells within 48 hr after the phagocytosis.

Table I. Phagocytic Uptake of NR8383 Cells Toward Live BCG and h-kBCG

	Phagocytic Population of Mϕ Cells (%)[a]			
	Number of BCG Phagocytosed[b]			
Incubation Period (h)	2	4	6	10
Live BCG	15.2±0.8	17.7±2.3	27.7±2.3	26.3±1.3
	2.14±0.55	2.38±0.48	2.61±0.97	2.78±0.84
h-kBCG	16.5±5.8	20.5±4.1	25.7±2.1	26.8±3.2
	1.90±0.39	2.87±0.78	2.88±0.97	2.81±1.15

Values are means±SD from at least three separate runs.
[a] Populations of phagocytic NR8383 cells toward live BCG and h-kBCG were determined at an MOI of 10 after incubation for various periods at 37°C. Phagocytic Mϕ cells were counted among a total of 200 Mϕ cells by light microscopic observation at magnification ×1,000.
[b] The mean numbers of the BCGs phagocytosed per individual Mϕ cell.

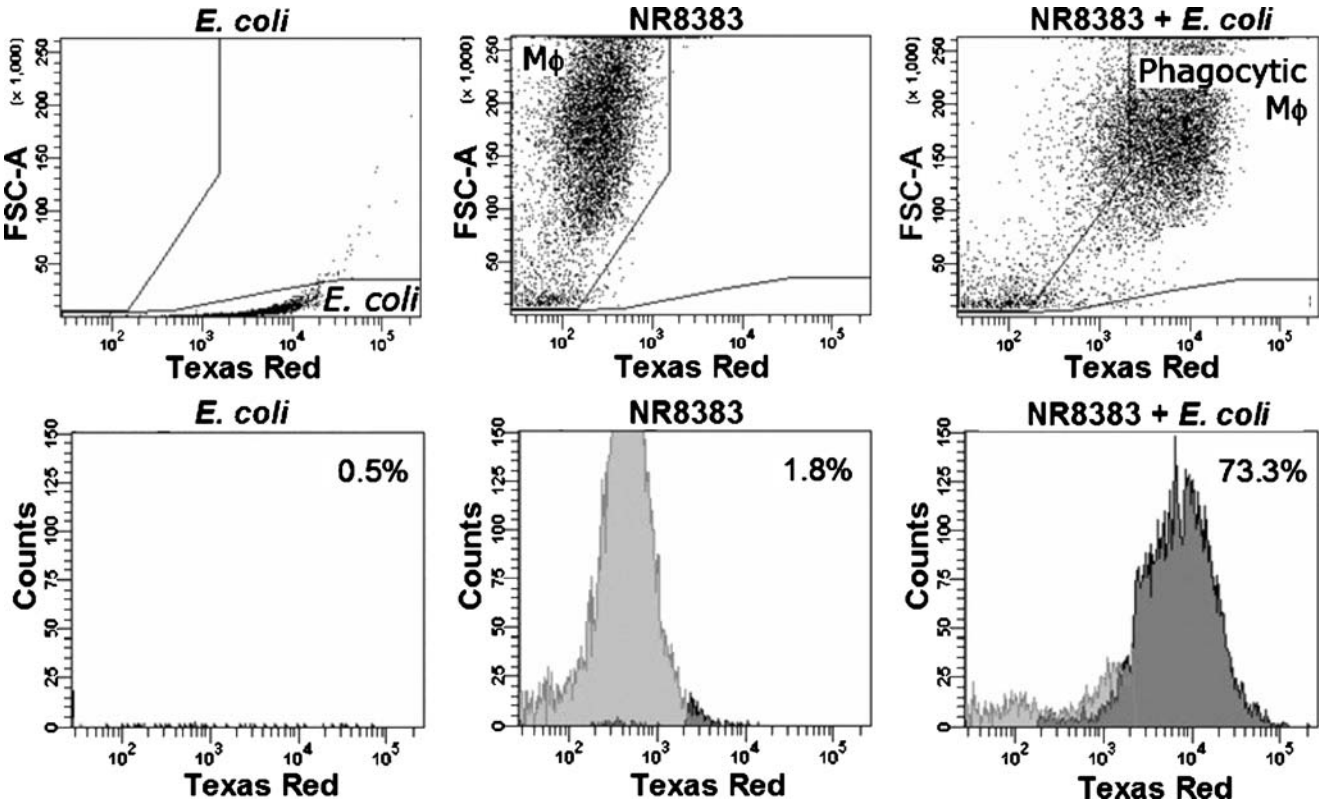

Fig. 2. Dot–plot profiles and histograms of flow cytometric analysis for phagocytic uptake by NR8383 cells of *E. coli* labeled with Texas Red. Dot–plot profiles (*top*) and histograms (*bottom*) of *E. coli* (*left panels*), intact NR8383 (*center panels*), and NR8383 cells incubated with *E. coli* at an MOI of 10 for 10 h (*right panels*) show that the population of Mϕ cells that phagocytosed *E. coli* could be distinguished from that of Mϕ cells that did not phagocytose them. The population of the phagocytic Mϕ cells was determined by the shift of the fluorescence intensity of the non-phagocytic Mϕ cells (*light gray histograms*) to that of the phagocytic Mϕ cells (*dark gray histograms*).

TNF-α Production Induced by Phagocytosis of Microorganisms

As shown in Fig. 5, phagocytosis of live BCG did not produce TNF-α in alveolar Mϕ cells in 6 h, but it produced 600 pg/ml of TNF-α after incubation for 24 h, and this concentration remained almost constant until 48 h. Phagocytosis of h-kBCG also did not induce TNF-α production by 6 h, and the TNF-α level increased steadily with time for 48 h

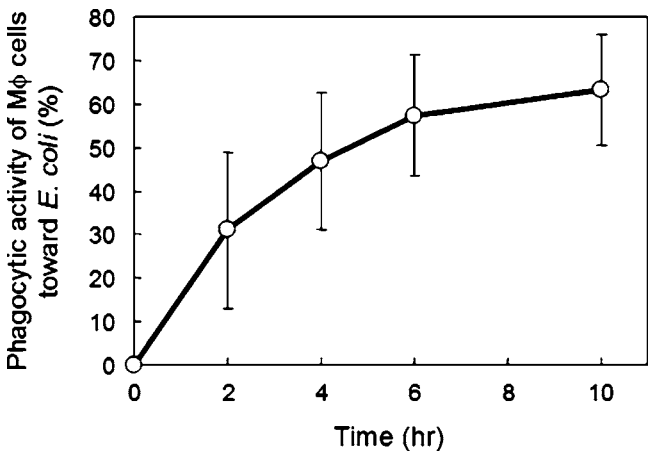

Fig. 3. Phagocytic uptake of *E. coli* by NR8383 cells. NR8383 cells were incubated with *E. coli* at an MOI of 10 for various periods at 37°C. Phagocytic activity was determined in terms of the relative population of the phagocytic Mϕ cells (%) determined by flow cytometric analysis of approx. 10,000 Mϕ cells. Values are means±SD from at least four separate runs.

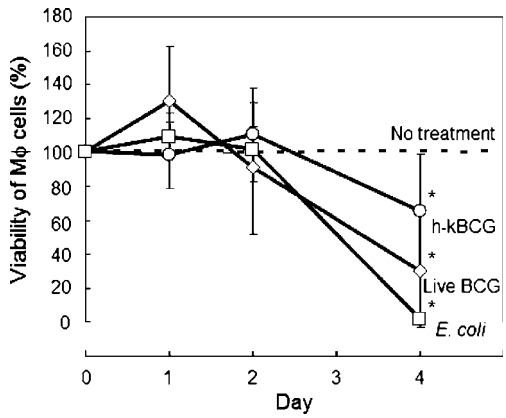

Fig. 4. Viability of Mϕ cells after uptake of microbes. NR8383 cells were incubated with either live BCG, h-kBCG or *E. coli* at an MOI of 10 for various periods at 37°C. Viability of Mϕ cells was determined by conducting the WST-8 assay described in "Materials and Methods", and was expressed as a percentage relative to the total number of NR8383 cells used in the phagocytic experiments. Values are means±SD from at least five separate runs. Results for treatment with live BCG, h-kBCG, and *E. coli* are shown by *open diamonds*, *open circles*, and *open squares*, respectively. The *asterisk mark* indicates statistically significant comparison ($p<0.05$) by the Student's *t* test.

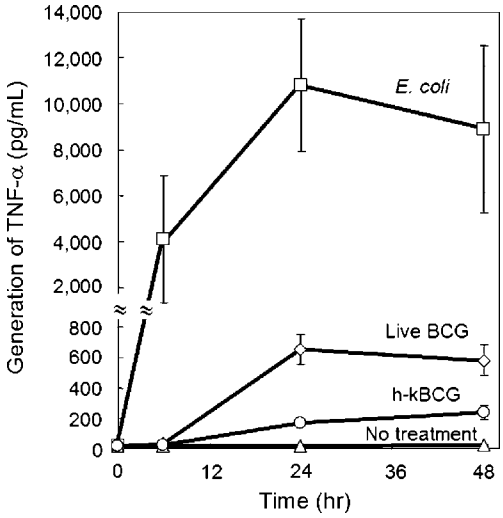

Fig. 5. Release of TNF-α from NR8383 cells induced by treatment with microbes. NR8383 cells were incubated with either live BCG, h-kBCG or *E. coli* at an MOI of 10 for various periods at 37°C, and then the amount of TNF-α released into the incubation medium was determined by ELISA. Experiments were performed in separate three runs, each of which consisted of duplicate assays; and the results are shown as the means±SD. *Open diamonds* represent the results with live BCG; *open circles*, with h-kBCG; *open squares*, with *E. coli*; and *open triangles*, without microbial treatment.

to attain the TNF-α concentration of about 250 pg/ml, being less than half of that induced by live BCG.

In contrast, *E. coli* generated TNF-α instantly after the start of their incubation with Mϕ cells, and the TNF-α level increased sharply with time of incubation until 24 h, at which time it reached the maximal level of approx. 11,000 pg/ml. Namely, the amount of TNF-α induced by *E. coli* was more than 15 and 40 times greater than that induced by live BCG and h-kBCG, respectively. After 24 h, TNF-α production decreased slowly. It is interesting that the release pattern of TNF-α from alveolar Mϕ cells was dependent on the microbes phagocytosed. The activation of alveolar Mϕ cells by phagocytosis of either type of BCG was characterized by a lag time and low production of TNF-α. In contrast, that triggered by the phagocytosis of *E. coli* was instant and high production of TNF-α ensued. No TNF-α was produced from Mϕ cells not incubated with any microbes.

NO Generation After Phagocytosis of Microbes

On phagocytosis of mycobacteria, reactive oxygen species (ROS) and nitric oxide (NO) are generated, and they affect phagocytic activity of Mϕ cells. The effect of NO might be consistent due to its steady generation mediated by inducible NO synthase, but that of ROS is regarded to be transit due to its short life-time (25). In addition, it is suggested that the effect of NO generated by infection with MTB is more influential than that of ROS on the phagocytic activity of Mϕ cells (26). Hence, we examined the NO production on phagocytosis of microbes.

We determined the nitrite with Griess reagent, as the nitric oxide (NO) generated is instantly oxidized to nitrite (27). The value of nitrite was taken as that of NO in this paper. As shown in Fig. 6, alveolar Mϕ cells that phagocytosed live BCG and h-kBCG generated NO to similar extents throughout the incubation period. Namely, NO was generated after a lag time of 6 h; and its concentration induced by either BCG was almost the same, being approx. 10 μM after 24 h and 35 μM after 48 h. The production of NO triggered by the phagocytosis of *E. coli* was much greater than that by the BCGs, although a 6-hr lag occurred prior to the generation of NO, as in the case of the BCGs. The NO concentration at 24 h was 40 μM, being equivalent to that generated by phagocytosis of either type of BCG at 48 h, and it increased to approx. 80 μM after incubation for 48 h. In contrast, no NO was produced even up to 48 h from Mϕ cells not exposed to any bacteria.

Effect of Phagocytosis of Microbes on Phagocytic Activity of Alveolar Mϕ Cells Toward Artificial MS

We examined the effect of phagocytosis of live BCG, h-kBCG or *E. coli* on the phagocytic activity of alveolar Mϕ cells toward cPLGA and PSL MS. The phagocytic activity of approx. 10,000 Mϕ cells was analyzed with a flow cytometer. The dot–plot profiles in Fig. 7 showed that the Mϕ cells that had phagocytosed these MS were well separated from the Mϕ cells that had not taken up these MS.

The effects of phagocytosis of microbes on the phagocytic activity of Mϕ cells toward artificial MS were determined as a function of the incubation time of the infected Mϕ cells after phagocytosis of microbes. As shown in Fig. 8, the phagocytic activity of the Mϕ cells toward cPLGA MS was stimulated by phagocytosis of live BCG and h-kBCG, and the population of the Mϕ cells that phagocytosed cPLGA MS increased up to 60–70%, which corresponded to 1.3–1.5 times greater than that of Mϕ cells without treatment with microbes. This level held for 24 h, and then slightly decreased. It is noteworthy that the phagocytosis of both types of BCGs affected the phagocytosis of cPLGA MS at least for 48 h, at which time the phagocytic activities toward cPLGA MS were both 56%, being still somewhat greater than the phagocytic activity seen without microbes. In contrast, the phagocytosis of the *E. coli*-treated Mϕ cells toward cPLGA MS decreased consistently for 48 hr, dropping from 48 to 15%.

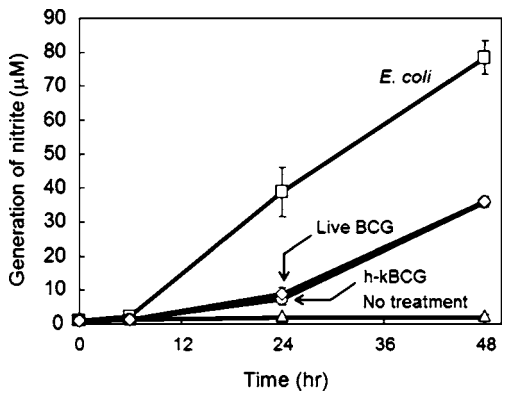

Fig. 6. Release of NO from NR8383 cells induced by treatment with microbes. The amount of NO was determined in terms of nitrite by using the Griess assay. Experimental conditions were as described in the legend for Fig. 5.

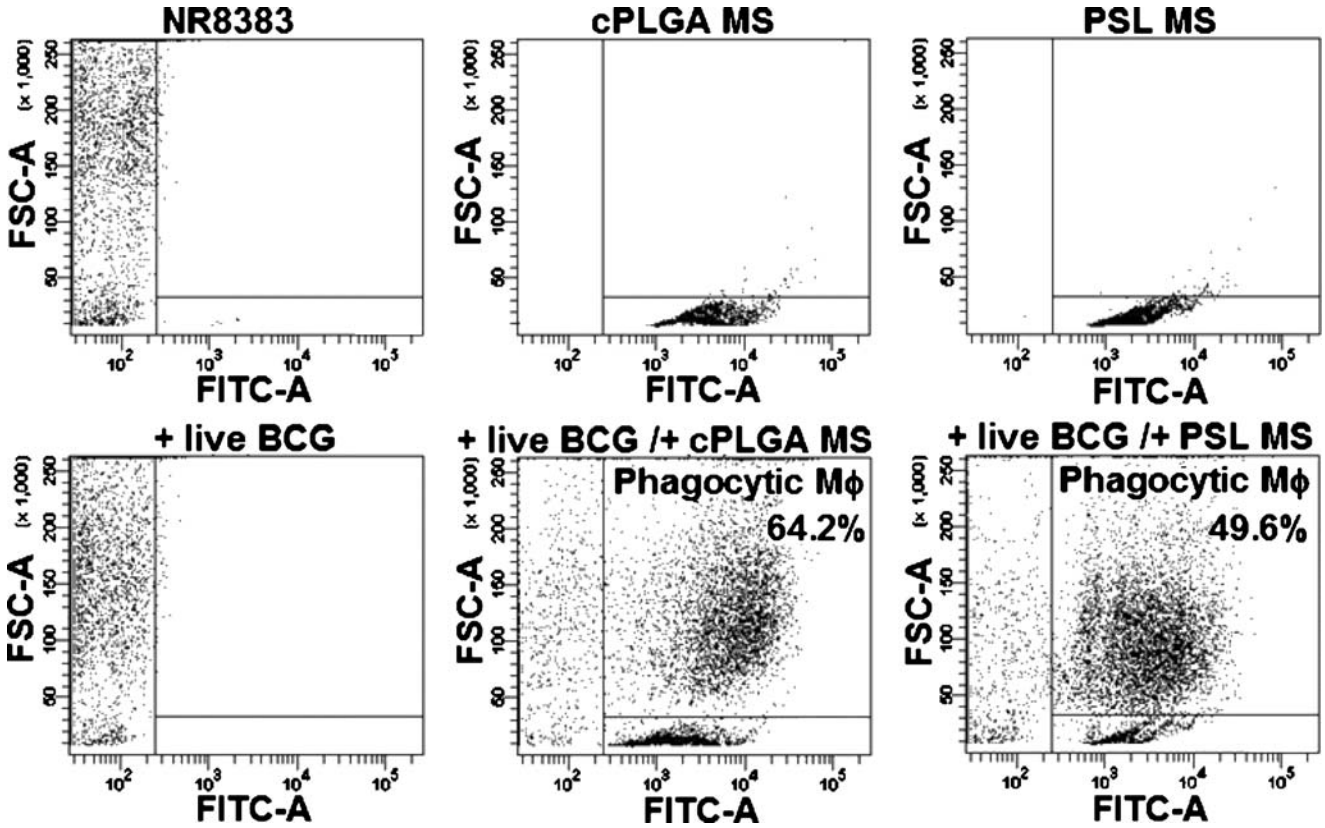

Fig. 7. Dot–plot profiles of phagocytosis toward synthetic polymer MS by NR8383 cells previously infected with live BCG. NR8383 cells were incubated with live BCG at an MOI of 10 for 6 h at 37°C. After separation of extra Mϕ cellular BCG, the Mϕ cells were incubated for 2 h with cPLGA or PSL MS. The phagocytic population was then assayed with a flow cytometer, and the results are shown as dot–plot profiles. *Left panels*, intact NR8383 (*top*) and infected NR8383 (*bottom*); *middle panels*: cPLGA MS only (*top*) and infected NR8383 incubated with cPLGA MS (*bottom*); *right panels*: PSL MS only (*top*) and infected NR8383 incubated with PSL MS (*bottom*).

We next examined the effect of infection with live BCG on the phagocytic uptake of the RFP-PLGA MS by NR8383 cells. For this, we incubated NR8383 cells with live BCG for 6 h, and then they were treated with RFP-PLGA MS for 2 h. About 40% of NR8383 cells phagocytosed live BCG, and the population of Mϕ cells that phagocytosed RFP-PLGA MS was about 60%. Then, population of the phagocytic Mϕ cells was determined for BCG-infected and un-infected Mϕ cells separately by light microscopic observation. As shown in Fig. 9, more than 70% of the infected NR8383 cells phagocytosed RFP-PLGA MS, but about 48% of the un-infected Mϕ cells took up the MS, the latter value being similar to that (about 50%) for Mϕ cells without pre-incubation with BCG. These results suggest that infection of alveolar Mϕ cells with live BCG is advantageous for the phagocytic uptake of RFP-PLGA MS.

Like the effects on phagocytosis toward cPLGA MS, phagocytosis of PSL MS was activated by uptake of either live BCG or h-kBCG, but was suppressed by *E. coli*, as shown in Fig. 10. However, the effects were more significant than those for cPLGA MS. The population of Mϕ cells that phagocytosed PSL MS without treatment with microbes was 28%, and it increased to more than 50% upon uptake of either type of BCG (time period 0 in Fig. 9). This level fluctuated slightly with prolonged incubation of the Mϕ cells that had phagocytosed BCGs, but held almost constantly for 48 h. In contrast, treatment of Mϕ cells with *E. coli* suppressed the phagocytosis toward PSL MS. This suppressive effect increased with time of incubation and leveled off at the value of 5% after 48 h.

DISCUSSION

Phagocytosis of MTB is known to trigger the production of TNF-α and NO in alveolar Mϕ cells (7–11), and they affect the phagocytic activity of Mϕ cells (14,15). The unknown effects of phagocytosis of MTB on the phagocytic uptake of RFP-PLGA MS should be examined, because the effects would be of importance for clinical treatment of tuberculosis by pulmonary inhalation of RFP-PLGA MS. Hence, we studied the effects of live BCG on the phagocytic activity of alveolar Mϕ cells toward artificial microspheres in comparison with those of h-kBCG and *E. coli*.

Previously, we reported that the populations of alveolar Mϕ cells that took up RFP-PLGA and PSL MS at an MS/Mϕ (corresponding to MOI) of 10 for 4 h were more than 80 and 60%, respectively (19). We found in this study that phagocytic activities of alveolar Mϕ cells toward BCGs were significantly lower than those toward synthetic polymer microspheres, but that toward *E. coli* was comparable to that toward synthetic microspheres. Another difference between phagocytosis of microbes and synthetic polymer microspheres was the period necessary for attaining the saturation level of phagocytosis; about 6–10 h were necessary for alveolar Mϕ cells to attain

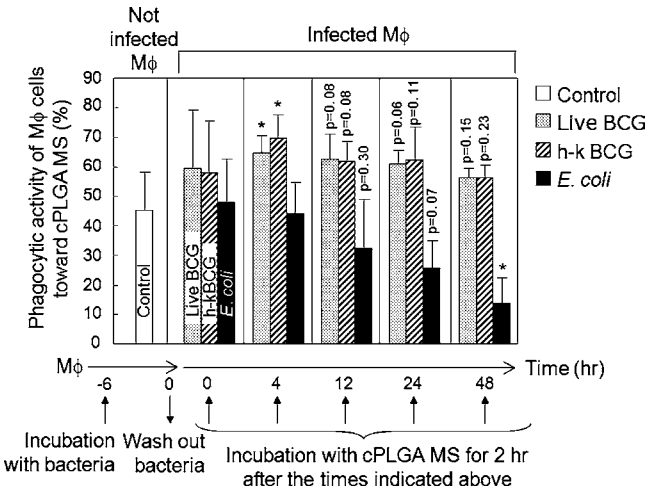

Fig. 8. Effect of infection with microbes on phagocytic activity of NR8383 cells toward cPLGA MS. NR8383 cells were incubated with either live BCG, h-kBCG or *E. coli* at an MOI of 10 for 6 h at 37°C. After the phagocytic Mϕ cells had been washed to remove non-phagocytosed bacteria and then incubated for various periods shown in the figure, they were incubated with cPLGA MS at an MS/Mϕ of 10 for 2 h. Thereafter, the Mϕ cells were subjected to flow cytometric analysis. Values are the means±S.D. from at least three separate runs. The phagocytic activity is represented as a percentage in terms of the population of Mϕ cells phagocytic toward cPLGA MS relative to the total of approx. 10,000 Mϕ cells analyzed by flow cytometry. Phagocytic activity of Mϕ cells toward cPLGA MS without microbial treatment is shown on the left hand side as a control. The *asterisk mark* indicates significantly different from control by the Student's *t* test ($p<0.05$).

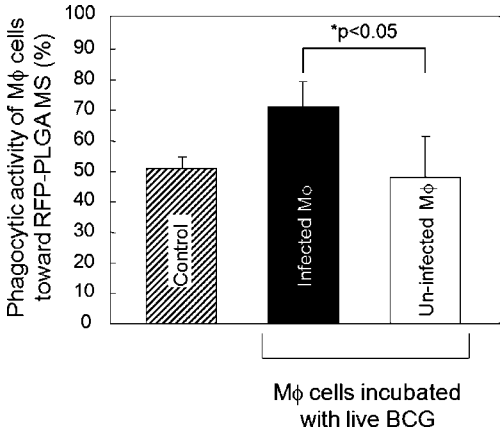

Fig. 9. Effect of BCG infection on the phagocytic uptake of RFP-PLGA MS by NR8383 cells. NR8383 cells were incubated with live BCG at an MOI of 10 for 6 h at 37°C, and Mϕ cells were washed to remove BCG that had not been phagocytosed. Then, NR8383 cells were incubated with RFP-PLGA at MS/Mϕ of 10 for 2 h. The phagocytic activity in terms of the population of Mϕ cells that phagocytosed MS was determined separately for infected Mϕ cells and Mϕ cells without infection by counting the population of the phagocytic cells that phagocytosed RFP-PLGA MS under a light microscope for total 200 cells. The result for NR8383 cells without pretreatment with BCG is shown as a control. Values are the means± SD from at least three separate runs, the *asterisk mark* indicates statistically significant comparison ($p<0.05$) by the Student's *t* test.

their maximal level of phagocytosis toward microbes, whereas approx. 4 h was adequate in the case of synthetic polymer microspheres (19,20,28).

The opsonization of MS with serum component affects the phagocytic activity of alveolar Mϕ cells. However, this effect will be not great for alveolar Mϕ cells, because they reside on the outer surface of alveoli, where there are quite few serum components. We found that the phagocytic activity of NR8383 cells toward RFP-PLGA MS in the presence of 4% FBS was almost the same as that without FBS, suggesting that there is no appreciable opsonization effect of FBS on the phagocytic activity of NR8383 cells toward cPLGA MS and PSL MS. In addition, the size and surface potential of the particles are in general regarded to affect the phagocytic activity (19,29,30). Actually, a particle size between 3 and 6 μm of RFP-PLGA and PSL MS are suggested to be optimal for phagocytosis by NR8383 cells (19,28). The RFP-PLGA MS, having almost null surface charge, are more advantageous for phagocytosis by Mϕ cells than PSL MS, which have a large negative surface charge of −35 mV at pH 7.4 (19). In contrast, both BCGs and *E. coli* are reported to carry negative charges of −50 and −30 mV, respectively, at approx. pH 7 (31,32), and their sizes are 1–4 and 2–6 μm, respectively, suggesting that the higher phagocytic activity toward *E. coli* than toward BCGs was due at least in part to the lower negative surface charge and favorable size of *E. coli.* Further study is necessary for understanding of the mechanism of more efficient phagocytosis of *E. coli* than BCG by alveolar Mϕ cells.

We found that phagocytosis of live BCG, h-kBCG or *E. coli* did not affect the viability of alveolar Mϕ cells within 2 days after phagocytosis. However, it did decrease the viability after 4 days, the effect being most significant with *E. coli.* The effect of *E. coli*, which caused death of all Mϕ cells, was likely due to the caspase-mediated apoptosis of Mϕ cells upon ingestion of *E. coli* (33). Live mycobacteria are also known to induce apoptosis in Mϕ cells, but this apoptosis is mediated by a caspase-independent pathway (34,35). However, Mϕ cells treated with the digestible h-kBCG did not undergo appreciable apoptosis.

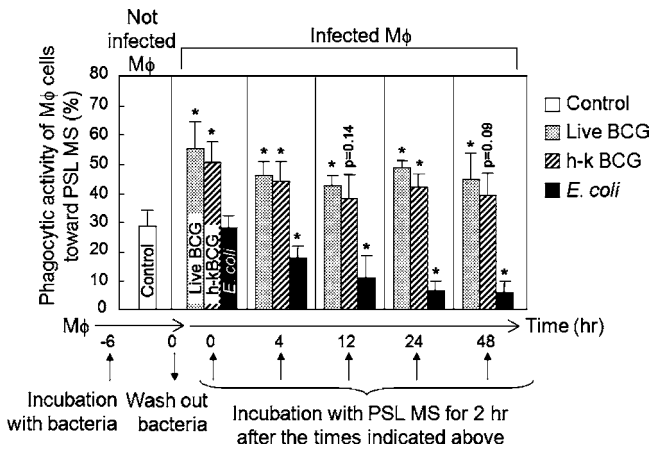

Fig. 10. Effect of infection with microbes on phagocytic activity of NR8383 cells toward PSL MS. Experimental conditions were essentially identical to those described in the legend of Fig. 8, except that PSL MS were used instead of cPLGA MS.

Phagocytosis of microbes induced the generation of TNF-α and NO in Mϕ cells. A large amount of TNF-α was released instantly upon phagocytosis of *E. coli*, whereas the production of it following phagocytosis of either type of BCG required a lag time and was very low. TNF-α production is mediated by toll-like receptors (TLRs), which recognize microbial components such as lipopolysaccharide and lipoarabinomannan. In particular, mycobacteria is recognized by TLR2; and the lipopolysaccharide of *E. coli,* by TLR4 (16,36). Hence, TNF-α generation initiated by signaling from TLR4 by *E. coli* should be more efficient than that by BCGs derived from the signaling from TLR2. In addition, the generation of TNF-α derived from TLR2 may require a certain lag time, but that from TLR4 does not, as was observed in this study. It is interesting to note that TNF-α induced by h-kBCG at 48 h was about 1/20 of that induced by live BCG. Higher TNF-α generation by live BCG could be due to the stimulation of endocytic network by the cell-wall glycolipids of live BCG (37), but the cell-wall constituents are altered by heat treatment (38).

Generation of NO was triggered by TNF-α accompanied with phagocytosis of microbes (39,40). The lag time in the NO generation derived from the phagocytosis of BCGs and *E. coli* should be the time required for the production of NO in response to the signal transduction triggered by TNF-α. It is thus reasonable that the generation of NO would be higher due to the higher TNF-α level induced by phagocytosis of *E. coli* than that resulting from the lower production of TNF-α induced by BCGs. The phagocytosis of live BCG caused NO production to a similar extent to that induced by h-kBCG, even though the level of TNF-α production by treatment with live BCG was more than twice that by h-kBCG. Nitrite production by h-kBCG was partly derived from the h-kBCG constituents digested with lysosomal enzymes in Mϕ cells (41). Hence, the actual amount of NO released from Mϕ cells by h-kBCG should have been less than that determined in terms of nitrite, possibly leading to a lesser killing effect on Mϕ cells due to apoptosis induced by NO (42,43). This could be a reason why the viability of NR8383 cells treated with h-kBCG was much higher than that of the cells treated with live BCG. This interpretation is supported by the result that immunization using h-kBCG did not induce an inducible NO synthase in mice, whereas that using live BCG did (44).

It is noteworthy that the phagocytic activity of alveolar Mϕ cells toward both artificial MS was increased by preincubation with either live BCG or h-kBCG for 6 h. It is possible that an increase in the cytoskeletal rearrangements, such as cell spreading induced by BCGs, might have enhanced the collisional frequency of MS with Mϕ cells (45). In addition, it is reported that phagocytosis of BCG induces up-regulation of scavenger receptors in Mϕ cells and that the negative charge of PSL MS is responsible for recognition by these scavenger receptors (46–49). Thus, it would be reasonable that the increase in the phagocytic uptake of PSL MS by pretreatment with BCG was more than that of the electrically neutral cPLGA MS. In our preliminary experiments, the phagocytosis of RFP-PLGA MS did not generate appreciable amounts of TNF-α and NO. Hence, these MS did not activate NR8383 cells. Interestingly, we found that the phagocytosis of BCG-infected Mϕ cells toward RFP-PLGA MS was greater than that of the Mϕ cells that had not been infected with BCG, and that the population of the un-infected Mϕ cells was the same as that of the Mϕ cells without exposure to BCG. Hence, the phagocytosis of BCG by alveolar Mϕ cells stimulates the phagocytic uptake of RFP-PLGA MS. In contrast to the effect of phagocytic uptake of BCGs, treatment of Mϕ cells with *E. coli* inhibited the uptake of both cPLGA and PSL MS. This could be due to generation of a large amount of TNF-α and NO by the phagocytosis of *E. coli*.

In conclusion, the phagocytosis of both types of BCGs by alveolar Mϕ cells induced lesser effect than that of *E. coli* on the viability of Mϕ cells, and activated the phagocytic uptake of PLGA and PSL MS, whereas that of *E. coli* inhibited the uptakes significantly, possibly due to much lower production of TNF-α and NO than that generated by phagocytosis of *E. coli*. These features should be of importance to the inhalation of PLGA MS laden with antitubercular agents for the clinical treatment of tuberculosis.

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